

Students on the poverty line

The British government will this week be forced to back down from last month's proposals for modifying students' grants. It should take the chance to reform the system.

SIR Keith Joseph's attempt last month to provide a modicum of help for the British academic research enterprise seems to have blown up in his face. The plan (see *Nature* 15 November, p.185) was to add £14 million to the income of the British research councils, and to give the University Grants Committee an extra £10 million to spend on equipment, financing the operation within the government's ceiling on public spending by reducing the value of the maintenance grants paid to university students. What Sir Keith seems to have overlooked, and what his Cabinet colleagues failed to spot when they approved his part of last month's package of public spending plans, is that the uniquely British system of university maintenance grants has become part of the British way of life, and part of the middle-class way of life in particular. By last weekend, the government was in serious trouble. Nearly half of its back-bench supporters in the House of Commons had declared themselves against the new arrangements, much to the satisfaction of the government's formal opponents. Most British newspapers have denounced the changes planned in language which is a rich blend of invective and humbug. But even the president of the Royal Society, speaking at the society's annual dinner last Friday with Sir Keith Joseph sitting as his guest a few feet away, offered the opinion that while the extra £24 million for research would be welcome, he regretted that it had to be won at the expense of student grants. Students are no doubt surprised to find how many friends they have.

Joseph, the Secretary of State for Education and Science, will be less surprised by these demonstrations that he has so few friends. He has had a long run of unpopularity (first at what was then the Department of Industry) going back to 1979, at least in part on the dubious ground that he inclines to abstract argument, being a kind of theoretician among politicians. It would nevertheless be a shame if, during this hullabaloo about student grants, it is forgotten that, in two consecutive British budget years, he has done his best to win more resources for the support of research, last year by the simple device of asking for more (and being refused), this year by shifting funds from one line to another within his own department's budget. Latterly, the minister has also been shaping up to tackle the obvious problems of British secondary education (specialization and the persistence of sloppiness) in ways from which his predecessors have mostly shrunk. Against the odds of a few years ago, it would be a misfortune if the government seeks to solve its immediate difficulty (how to back down without seeming to do so) by executing the traditional penalty for the bearers of bad tidings, for example by sending Sir Keith Joseph off to the House of Lords.

Restrictions

What the fuss this week should be about is not, in any case, the particular amendment of the students' maintenance scheme put forward by the minister but the scheme itself, which is a monument to the optimistic 1960s, when the expansion of British higher education was set in train. The principle is that young people should not be deterred from seeking higher education by the cost of doing so, for which reason local authorities are required by law to pay the tuition fees of British students at universities, polytechnics and other higher education establishments, and also to provide them with a grant to cover the cost of maintenance which, this year, ranges from £205 a year to nearly

ten times as much, depending on parental income. One of the consequences of this arrangement (which allows local authorities to reclaim the cost of grants from central government) was the imposition in 1981 of iniquitous and uneconomic quotas restricting student numbers at universities. The changes now proposed are adjustments to the way in which student grants are calculated from parental income which will have the effect of giving less to students with parents whose income exceeds £13,000 a year (after certain deductions). The new proposals do, however, break new ground in that they will require the offspring of well-off parents (with combined incomes above £20,000 a year) to contribute towards tuition fees (up to a maximum of £520 a year). The new scales will apply to all students in higher education at the beginning of the next academic year, those already embarked on academic careers included.

Hardship

This ramshackle system will be made only marginally more inequitable by the changes now proposed. So long as "mandatory" grants remain, British governments will feel compelled to hold opinions about the numbers of students higher education establishments should be teaching (which, strictly speaking, is none of their business). At the same time, the numbers of students suffering financial hardship will be increased as greater numbers of parents decline to make the financial contribution that the system implies is needed. And the proportions of young people from poor homes seeking higher education of any kind is likely further to decline, which is ridiculous at a time when everybody (the government included) agrees that too small a proportion of young people in Britain are acquiring a modern skill. The remedy for all these evils will be found not by tinkering with the present system but by redesigning it. This is how the government should set about that task.

First, to put out the immediate fires, the distasteful retrospective element in the new proposals should be dropped; students part way through a course should be allowed to complete it under the rules understood when they began. But the government should also recognize that the sums of money it is assuming middle-class families in impoverished Britain will find to put their sons and daughters through higher education will for many be substantial fractions of an annual income. Unfortunately, after a long flirtation with the idea, the government has now set its face against proposals for loan schemes to help finance higher education; it should now reconsider that decision. It should go further, and acknowledge that one of the reasons for this week's middle-class revolt is that many people have suddenly woken up to what they should have known all along, that contrary to the appearance suggested by the mandatory grants scheme, British higher education is not (and has not been for a long time) free. What needs now to be done is to hammer out an equitable basis for meeting the costs.

The first need is for a decision on tuition fees. Should British students pay, or be excused? The way in which this question is answered is vital for the health of the university system as well as for the welfare of students, for fee income is potentially one of the ways in which British universities can hope not to be entirely dependent financially on the government. But what passes for a "tuition fee" at present is an entirely notional sum of money



which is, to what should be everybody's surprise, fixed not by universities but by the government (which repeatedly adjusts it up when it wishes to augment universities' sense of freedom and down, as in 1981, when it wishes to cramp them in). What would now best suit the universities is an understanding that they should be free to charge what tuition fees they choose, and that they should keep what they collect. Ideally, the fees asked of students should be related to the marginal cost of education (and not to the average, which must be substantially greater), but if the years ahead may see some universities hard pressed to find students, they may need the freedom to charge what the market will bear.

The question of whether governments should, in principle, pay the cost of maintaining students in higher education is more complicated, and goes back to the report of the Robbins committee which, in 1963, launched the expansion of the British university system with the doctrine that access should be open to all school-leavers who are qualified to take advantage of it. Especially because so many of the new British universities are sited miles away from their catchment areas, the assumption has grown up that students will ordinarily travel to some distant place if they enter higher education, and successive governments have found themselves embroiled in tedious arguments with the National Union of Students over the proper level of the "travel grant" by which the ordinary maintenance grant is supplemented. One consequence, from which the British university system as a whole has suffered, is the erosion of the regional connections of universities such as the red-brick creations of the nineteenth century. It would not be a serious setback for the British system if the principle were now followed that student maintenance should not be a public charge. To protect universities that are vulnerable because of their geographical isolation, there would no doubt have to be a transitional arrangement of some kind.

Outside Britain, it is bound to seem strange that the provision of grants to students is a serious issue. Almost everywhere, students are constrained in their choice of higher education establishments by the costs not merely of tuition but of maintaining themselves independently. Can Britain, with its perpetual money troubles, afford to be different?

One practical consequence of such a move towards economic realism would be serious hardship among students, many of whom already live in too much squalor, perhaps waiting for parental contributions that do not materialize. Moreover, British students are less able than those elsewhere to earn money to help with their costs, partly because of the economic climate but also because of trade union restrictions, while most universities have no significant endowment funds with which to help able people in trouble. That is why British policy on students can be transformed to what it should be only by equipping universities financially to be able to do their duty to their students.

The problem of students from poor homes is different, serious but solvable. Entry to British higher education is still skewed against the children of the less well off, with the consequence that too little is made of the national pool of talent. The needs of these potential students are theoretically catered for by the present system of maintenance grants but, as the figures show, the outcome is inadequate. The simple remedy is to replace the present system by one of scholarships awarded to able school-leavers and others which, at their most generous, would cover not merely the bare cost of maintenance but some part of the opportunity cost of higher education (the wages forgone), but which would be tenable anywhere, not simply at the institution that happens to offer a student a place. Beyond the resolution of that problem there are other conundrums to be tackled. Slowly, in the past few years, British governments and universities have awakened to the need and opportunity for using existing educational plant for training persons of mature years, many of them already in jobs. But there are at present only disincentives (tuition fees out of taxed income, for example) to its efficient use. That is also a problem that needs solving. If the trouble Sir Keith Joseph has brought down on his government's head should have the effect of spurring a search for better ways of enlarging participation in higher education, it may yet be worthwhile. □

Japanese uncreativity

Japan's new strategy for research is more a recipe for continued success than for creativity.

JAPAN'S obsession with creativity is a curious phenomenon. Merely moderately successful nations are usually distinguished by their complacency. Japan, by contrast, after three decades of spectacular economic success, seems perpetually down in the dumps, with its conviction that Japanese technology is in reality derivative, and that its gigantic technical effort lacks creative flair. Is it possible that Japan's competitors in the West, beaten onto the ropes though they may be the technical achievements of Japanese industry, have been able to ensure that Japan does not fully enjoy its success by suggesting that there is something missing?

The point has been endlessly discussed in Japan in the past five years, but will not be settled with the publication this week (see facing page) of the blueprint for future research drawn up by the Council for Science and Technology in Tokyo. There is a grain of truth in the common Japanese assumption that many of the products with which Japan has swept the world are derived from machines already built elsewhere. But so what? From ships to video-recorders, Japanese technologists have been able to design and manufacture high-quality products more cheaply than others, and at the same time make sure that they function reliably. If the ingenuity is not creative, many technologists elsewhere will be wondering what is meant by creativity.

The more substantial sense in which Japan is technically uncreative is in the contribution of the university system to basic science, but the reasons are now familiar. First, the rigidity of the academic institutions, and the parsimony with which they are supported, drives energetic academics into applied science and into partnerships with industry. Second, students march predominantly into the academic engineering departments. Righting this imbalance would require long-due changes in the academic structure, which the science and technology council has plainly considered outside its terms of reference. Indeed, the list of objectives accompanying this week's strategic plan is not so much a recipe for creativity as a shopping list for tomorrow's high technology that could be found in most other places in the West. To be sure, Japan may succeed where others fail, but that is a very different matter. □

Damming the water

Are the British and French governments losing an opportunity?

WHAT follows is not a joke. For at least two hundred years, but at different times, the English and the French have been suggesting proposals for a permanent link across the 21 miles of water that separate them. Most schemes have turned on the construction of a tunnel. In the 1960s, bridges briefly became fashionable. Now, the favoured way of spanning the Channel is to build a composite structure, part bridge and part tunnel. To judge from the Anglo-French meeting in Paris last week, the remaining obstacles to a permanent link are merely legal and financial.

Nobody, however, has seriously considered what must be the most obvious way of spanning this narrow and shallow stretch of water, the building of a permanent dam across the Channel. The advantages are obvious (see *Nature* 219, 991; 1968). It would be possible to generate endless electricity, the Dutch would no longer have to worry about their dykes and it would be possible to walk across, as at the glacial maximum 18,000 years ago. The disadvantages are few, the most obvious of them being the disturbance caused to shipping, most of which could just as well go round the north of Scotland. The cost of such a project has never been determined, but could well turn out to be surprisingly small. Although megaprojects are now out of fashion, should not the British and French governments look seriously at this ideal before committing themselves to a clumsier alternative? □

Japanese research

Strategy for next decade aims at more creativity

Tokyo

THE Japanese government last week unveiled a major new report mapping out its fundamental policies for the promotion of science and technology over the next ten years. Stress is now being laid on the encouragement of creativity in science — apparently in response to Western criticism — and, along with it, greater international exchange and cooperation and the development of science in “harmony with human society”.

The report, entitled *Tackling changing conditions; Comprehensive and fundamental policies for long-term promotion of science and technology*, is the work of the Council for Science and Technology, the highest policy-making body in the nation, whose chairman is the Prime Minister. Some 150 experts drawn from the universities, government research laboratories and private industry helped to draw it up, together with the heads of the science-based ministries and the Science Council of Japan, the academic representative body, who have permanent seats on the council.

In its 24-year history, the council has produced only 11 reports. Each has tried to set a policy framework and to define key research areas so that the ministries concerned with science can implement detailed policy in accord with the government's overall aim.

To carry out the policies suggested by the new report, increased expenditure will be required — and increased expenditure certainly seems to be what Japan has been getting. The report reveals that Japan's research and development expenditure, most of it provided by private industry, will reach 3 per cent of national income this fiscal year (over 2.8 per cent of gross national product) after four years of growth at a remarkable 0.2 per cent of national income per year. Only West Germany among the industrialized nations can claim to be spending a greater percentage of its national income on research, but the lead should pass to Japan within 2–3 years.

In calling for increased creativity, the report accepts the view that “up to now Japan has taken in foreign science and technology, improved and developed it in a highly efficient response to social and economic needs to establish its present developed (industrial) state...”. But now the call is for measures to smooth the way for Japan to become one of the top nations in basic research. Several trends are seen as making this urgent: the so-called “soft revolution” — the accelerating pace of the information sciences, of which computer software development is one part, where Japan has not yet made a name for itself;

the ever closer links between basic and applied research required to make possible further “miniaturization” in electronics and sophisticated control of structure and function in materials; and the increasing influence science and technology are having on human life styles.

To increase creativity, better links are called for between university and industry — noticeably absent so far in Japan. No concrete measures are suggested, however, and it is difficult to see how the present trends towards increasing reliance on private sector research will be changed. Calls are made for more efforts to train researchers in biotechnology and advanced electronics in view of an expected shortfall that could limit basic research activities.

“Internationalization” is also called for — the first time a council report has clearly done so. Foreign participation in government research projects and foreign use of government facilities are possibilities mentioned. What the report does not mention, however, is that the current trend is towards increasing competition in basic research programmes (such as the fifth generation computer project) and attempts to launch cooperative projects at the various summits have not met with success: some revolutionary steps will be needed to bring about international cooperation on any significant scale.

In contrast with the generalities of much

of the report, which will require interpretation by the ministries concerned, key research themes where Japan must now make major basic research efforts are more clearly mapped out. These include research on new materials, electronics and information sciences, life sciences and earth sciences (see below). Many of the areas of materials sciences mentioned have already become the focus of long-term projects sponsored by the Science and Technology Agency and the Ministry of International Trade and Industry (see *Nature* 305, 373; 1983). Added here are calls for increased basic work on reaction theory and mechanisms to make possible the prediction of materials with new properties — welcome news to many university research groups.

In electronics and information sciences, areas likely to be commercialized in the near future are avoided. Stress is laid on fundamental projects such as production of the components necessary for optical circuits and eventually an optical computer, and steps towards a new science of “bioelectronics”. Several electronics companies have already moved into this futuristic field, setting up groups to research, in particular, Langmuir–Blodgett films.

Life science themes to be promoted include, unsurprisingly, all the fundamental technologies that will some day have applications in biotechnology. In the earth sciences, remote sensing and the building of new satellites and data networks lie at the heart of themes aimed to make better predictions of the natural disasters — earthquakes, typhoons and floods — to which Japan is prone, and to enable natural resources, including sources of geothermal heat, to be detected more easily.

Alun Anderson

Japan's strategic objectives

AMONG the basic research themes defined the report are:

Materials science

Materials produced under extreme conditions such as ultrahigh pressure, temperature and magnetic fields-strength; artificial lattice and amorphous structures; surface properties and new ways of analysing them; interaction of materials with X-ray, heavy and light ion beams; development of free-electron, excimer and chemical lasers; materials with mixed atomic and molecular bonding with new properties (organic superconductors); materials design by studies of unstable reaction intermediates and bonding properties, including development of associated computer stimulation and database facilities; control of form and purity as in ultra-fine particles, highly ordered structures and films; composite materials and molecular hybrids; materials mimicking natural materials, such as artificial bone; new analysis and evaluation techniques.

Information sciences

New devices, concentrating on Josephson

junctions, image processing and intelligent sensor devices; optical electronics; bioelectronics and biocomputing; knowledge management; fifth-generation computing and its connections with mathematics, symbol manipulation and psychology.

Life sciences

Gene regulation extended to cell regulation; extraction, analysis and synthesis of DNA; design, modification and synthesis of functional proteins; chromosome engineering, including modification and automatic mapping; cell and organelle modification; altering components of cells and tissues and development of the whole organism.

Earth sciences

Atmosphere–sea interaction, unusual weather conditions and typhoon generation, requiring new observation satellites and Earth-based networks; mantle dynamics, earthquakes and volcanic activity; new satellite-borne sensors and laser ranging equipment, new data networks and new Earth resource satellites.

Alun Anderson

Anglo-French cooperation

Technology dominates summit

AFTER the bitter battles over British contributions to the European Community budget and almost every other kind of acrimony between France and Britain, the British Prime Minister, Mrs Margaret Thatcher, and the French President, M. François Mitterrand, last week made up their differences at a meeting in Paris at which agreements on technology and research played an unusually prominent role.

M. Mitterrand was the cooler of the two. He spoke on Friday of "an excellent spirit of cooperation". Mrs Thatcher, more fulsome, spoke of "a completely new atmosphere... it is just a totally different bilateral from any we have had before... more constructive, more cooperative". With the budget issue out of the way, she wanted to suggest, real Franco-British cooperation can begin.

Technology came up as the list of major projects on which Britain and France have either agreed in the past 12 months or hope soon to agree. The highlight was the Channel Tunnel, or "fixed Channel link" as it is called in France to include the possibilities of both tunnel and bridge.

The two governments, no doubt aware that the tunnel was first suggested in 1751, emphasized the "urgency" of the studies now under way that will identify the political structures under which the "link" can go ahead. Both sides have agreed that the link must be paid for by private funds, but potential backers have asked for guarantees that construction will not be halted for political reasons.

The tunnel apart, the Franco-British summit raised the following issues:

- Esprit, the European cooperative research programme on information technology, which is felt greatly to have improved bilateral links between French and British companies.

- Fast reactor development, where outline agreements among European utilities, research organizations and fuel-cycle companies were reached this year following a political umbrella agreement in January. (But details of exactly who does what and where have still to be agreed.)

- The Versailles summit programme, under which "quite fruitful" Franco-British work is going on in biotechnology and food technology.

- A Chinese pressurized water reactor for Guangdong, under development jointly by Framatome of France and General Electric Company of Britain. "We hope, both of us, to get substantial orders for that power station", said Mrs Thatcher last Friday.

- Direct broadcast satellite (DBS) transmission standards, where bilateral discussions are well advanced to avoid the dual standards (PAL and SECAM) that have plagued the European domestic television market.

- The European A320 Airbus, on which Britain is to collaborate more fully than on the earlier A300 and A310 versions, it is claimed in Paris.

- A military helicopter engine (RTM 322) under joint development between Rolls-Royce and Turbomeca, a third-generation antitank weapon (with Germany) and a multi-launch rocket system (also with Germany).

Telecommunications and space projects were also mentioned, although progress in

opening the British telecommunications market to the French (and vice versa) has not gone as fast as the French would have liked, and space agreements must wait for the European space summit in Rome next January.

The meeting also postponed until next year a Franco-British "association" for discussing the exchange of results between universities and industry (see *Nature* 1 November, p.4) which will be debated at a bilateral meeting in February. Meanwhile, soundings are taking place to determine exactly what each side would like to get out of such an institution, and how it would work in practice.

Robert Walgate

Polish science budget

Paper generosity decreed

Warsaw

POLAND'S science budget, at present one of the lowest in Europe in relation to national income, is to be substantially increased. Last week, the academic secretary of the Polish Academy of Sciences, Dr Zdzisław Kaczmarek, told the presidium of the academy that next year the government would increase the academy's "science" budget by 26 per cent, and that financial support for the academy would be further strengthened by an allocation of 1,000 million zloty from the central technical economic fund. This, said Dr Kaczmarek, would allow more money to be allotted to research. Wages within the academy network, moreover, would be increased, on the average, to 10 per cent above the national mean, rising to 30 per cent above the national mean in the next three years.

Dr Kaczmarek's announcement has been received with a mixture of relief and

scepticism. Poland at present spends only 2.5 per cent of the national income on science (including the university and industrial research sectors), and any increase is clearly welcome. Many scientists, however, feel considerable doubt as to whether the wage rises can be as substantial as promised. One researcher said that her own monthly salary, 7,000 zloty, is not only far below the national average of 17,000 zloty, but well below the salary of those doing similar work in the universities.

There is also some scepticism about the new "Committee for Matters of Science and Technical Progress", which is intended to have responsibility for research areas of science of particular importance for the national economy. Many of the older generation of scientists see it as a return to a similar body of the 1960s, when the academy's powers were considerably less than at present.

Vera Rich

No cancer cure

Washington

A BATTLE is in prospect between the Food and Drug Administration (FDA) and the National Cancer Institute (NCI) over whether the government should throw the book at the Kellogg cereal company for its advertising campaign that promotes All-Bran cereal as a cancer preventive.

Kellogg's television and print advertisements running since the beginning of October state that "the National Cancer Institute [NCI] believes a high fiber, low fat diet may reduce your risk of some kinds of cancer", and then proceed to extol the virtues of All-Bran's high-fibre content. The print advertisements also tell readers how they can obtain additional cancer prevention information from NCI.

NCI officials, who have made diet modification a key component of their plans to cut cancer mortality by half by the year 2000, appear to support Kellogg's efforts; they particularly seem to like an offer by Kellogg to turn over to NCI the backs of their cereal boxes for additional

cancer-prevention messages. In a report to Health and Human Services Secretary Margaret Heckler, Assistant Secretary for Health Edward Brandt noted that "this effort by the Kellogg Company will infuse millions of dollars of private sector funds into the promotion of cancer prevention".

But FDA meanwhile has been looking



into the possibility that the Kellogg advertisements violate federal law. While avoiding direct comment on the Kellogg case, Mark Novitch, deputy commissioner of food and drugs, recently told a food industry meeting in Washington that "current FDA rules prohibit any labelling [on foods] that makes health claims".

Stephen Budiansky

Cohen-Boyer patent

Licensees ponder weaknesses

Washington

IN the aftermath of the long-awaited grant of the Cohen-Boyer patent in September, it is becoming increasingly clear that serious questions about the patent's validity remain unresolved. The opening of the complete file has in fact brought to light new questions, and Stanford University may now be reluctant to enforce its patent in the courts for fear of opening these issues to new scrutiny, possibly leading to judicial invalidation of the patent.

The September patent, which covers recombinant DNA molecules used in the cloning process developed by Stanley Cohen and Herbert Boyer, was issued to Stanford University and the University of California after a prolonged negotiation with the Patent Office. Stanford, which had initially waived its right to secrecy during this process, abruptly reversed course in November 1982 after the Patent Office tentatively rejected the claim and began asking uncomfortable questions.

It now turns out that shortly after asking that the file be closed to public scrutiny, Stanford's attorney, Bert Rowland, was told by the Patent Office that the case would be turned over to its fraud examiners; the examiners in spring 1984 concluded that Rowland and the applicants had committed a "clear violation of their duty to disclose to the Office information they are aware of which is material to the examination of the application". Because these violations were found to be the result of something less than bad faith or "gross negligence", however, they did not affect the ultimate issue of the patent.

The violations concern a scientific paper published by Cohen in 1977 that contradicts the earlier patent application description of how to make the key cloning vector pSC101, a plasmid. The Patent Office learned of the article only in June 1982, just as it was preparing to approve the patent; and the source of the information was press accounts of a critical article by Albert Halluin, now a patent attorney for Cetus Corporation. In the autumn of 1982, Rowland admitted to the Patent Office that he had known about the 1977 article when it had appeared, but had "no recollection" of why he failed to bring it to the Patent Office's attention. The fraud examiners' investigation further revealed that Rowland had written to Stanford's patent administrator in 1977 suggesting that the Cohen article might render the patent's description of how to make pSC101 "non-enabling" and asking whether they should revise the application accordingly.

Another apparent inconsistency is that in response to questions from the fraud examiners, Cohen early this year stated to the Patent Office that he had not appreciated the significance of the 1977 article with respect to the patent "until the

significance was explained to me at the time of my preparation of my declaration of April 20, 1983". In August 1982, however, Cohen denied reports in *Nature* (see 298, 595; 1982) and elsewhere that the 1977 paper admitted an error in the patent description. Rowland's declaration in autumn 1982 admitted to the Patent Office that pSC101 "could not be prepared in accordance with the working example" in the patent.

The real question for Stanford now is how many companies will continue to pay the \$10,000-a-year licence fee for the patent, due next February. Sixty-six companies are currently enrolled. The weakness of the patent is clearly weighing on the licensees' minds. An appellate court decision within the past month has con-

siderably toughened the definition of fraud, and if this standard had been applied to the Cohen-Boyer patent, it would probably not have been issued. The new tough standard would be applied by the courts now in any legal challenge to the patent's validity.

It is thought very unlikely that any company will go to the expense of mounting such a challenge. But Stanford itself would be opening the door if it goes to court to enforce the patent in an infringement action. It is thus likely that some companies may choose simply to ignore the patent, in effect daring Stanford to respond.

And even on the face of the patent claims, Stanford's position is not so strong as it would like it to be. The patent is limited to prokaryotes. And the admitted flaws in the pSC101 description remain in the earlier patent covering the cloning process, thereby raising doubts about that patent's validity.

Stephen Budiansky

Dangerous pathogens

Illegal US bug trade halted

Washington

TWO Canadians were arrested by Federal Bureau of Investigation (FBI) agents in Buffalo, New York State, last week as they attempted to collect botulinum and tetanus cultures that they had ordered under an assumed name from the American Type Culture Collection (ATCC). Neither of the two is a scientist, a fact that has fuelled speculations of a possible extortion scheme or terrorist plot.

The incident has also served to highlight the virtual absence of regulations governing the sale and acquisition of highly infectious or toxic agents. Federal regulations restrict only the importation or transfer of foreign microorganisms; indigenous microorganisms can in principle be sold freely to anyone. The two Canadians in fact obtained a tetanus culture from ATCC in September without incident.

It was only after they placed a second order that ATCC officials apparently became suspicious and approached FBI. The bureau found that the name and address used by the men were fictitious, and asked ATCC to process the order, substituting inert samples for live cultures. The Canadians were presumably going to carry the material across the border themselves, avoiding the customs problems of a direct shipment to Canada. When they attempted to pick up their order, FBI agents were waiting for them.

The only crime the two men have been charged with is wire fraud, for having used a false name in placing the order over the telephone. According to the FBI office in Buffalo, the men did not attempt to pass themselves off as scientists, although they did tell ATCC that they wanted the material for unspecified "research". FBI has identified the men as a Bell Canada employee and an Ontario Industrial Develop-

ment Corporation employee. No motive has been established.

ATCC is the major germ-plasm reference bank and supplies culture materials to researchers at or below cost; according to Pierre Daggett, acting director of ATCC, an internal safeguard system screens out people who are not qualified researchers and ensures that they do not receive infectious material. ATCC distributes 60,000 cultures per year. The regulations governing foreign microorganisms, which are administered by the US Department of Agriculture and the Public Health Service, require researchers to obtain a permit.

Both tetani and botulinum owe their lethality to toxins that they produce while growing under anaerobic conditions. Tetanus toxin is not normally harmful when ingested; botulin is a potent toxin when ingested or inhaled.

Toxin experts said last week that a botulinum culture of the type supplied by ATCC would probably be a mixture of spores and live cells, and possibly a small amount of toxin. Anyone with a basic knowledge of microbiological techniques, however, would have little difficulty in growing larger cultures and producing considerable quantities of toxin. On the other hand, any microbiologist would also have little difficulty in isolating botulinum or tetani from the environment.

Dr Michael Gill of Tufts University discounted the possibility that botulin could be used as a terrorist weapon: "The old scenario of putting it into a reservoir is not going to work". The bacteria would not grow in oxygenated water. But speculation is now centring on a possible extortion scheme, in which commercial products would be contaminated, or perhaps merely the claim made that this had been done.

Stephen Budiansky

UK research councils

Cake-cutting done

THE science and medical research councils are the chief beneficiaries of the extra £14 million the British government has made available for the coming financial year. The allocation of the extra funds was to be disclosed in the annual "forward look" of the Advisory Board for the Research Councils (ABRC), whose publication has been brought forward to help the Secretary of State for Education and Science to defend himself against his critics in the House of Commons who are complaining at the way the extra science money has been quarried from the budget for students' maintenance grants.

The ABRC document has something of the character of a ritual dance. To begin with, it records its own estimate of the funds that would be needed to take advantage of new opportunities for research that have been presented in the past year, reckoning that these would cost an extra £35.5 million in 1985-86. On the other hand, ABRC says elsewhere in the same document, the extra £14 million for next year (which is £16 million if account is taken of the way in which the government compensates the research councils for pay settlements) "will allow many important research projects" to continue that would otherwise have been allowed to lapse.

Much of the extra money for the coming financial year will be spent on what is called "restructuring", a euphemism for persuading researchers to retire early when the institutes at which they work are closed. ABRC had asked for £9 million in 1984-85, but the government has allowed £6 million (reducing to £5 million and £3 million in the two succeeding years).

Under the allocation scheme proposed by ABRC and accepted by the government, the medical and science research councils will benefit chiefly because they would otherwise have been "taxed" so as to free funds for other research councils having to close establishments. In reality, the Science and Engineering Research Council (SERC) will do best out of the new deal; its budget is due to increase from £279 million this year to £299.1 million (at 1984 prices) next year and to £306 million and £311 million in each of the two succeeding years. The Medical Research Council's budget will increase by £6 million between this year and next and then by a further £5 million in the two succeeding years. The budget of the Agricultural and Food Research Council is due to decline gently from next year, as forecast in previous forward looks.

Among the innovations of the new British science budget is a device whereby ABRC will keep up its sleeve (in the year beginning in April 1986) a contingency reserve, initially £6 million but doubling in the following year. At the same time, the cost of providing a secretariat for ABRC and of carrying out a few rudimentary

science policy studies will cease to be laundered through the budget of the Economic and Social Research Council, but will be identified separately.

The overall effect of the new proposals will on balance be neutral. Sir David Phillips, chairman of ABRC, says in the documents to be published this week that the extra money "will restore level funding". By the same test, the new arrangements will not make it possible to do much that is new. Nor will they help SERC and the British government to decide whether or not to pull out of CERN, the next big row in the research councils' calendar. □

EPA moves

Washington

PRESIDENT Reagan has named Lee M. Thomas to succeed William D. Ruckelshaus as administrator of the US Environmental Protection Agency (EPA).

Ruckelshaus, whose resignation takes effect on 5 January, has earned widespread praise for restoring morale at EPA following the scandals during the term of office of Anne M. Burford. Only one of the 20 political appointees who ran EPA under Burford remains today, the remainder having resigned, been fired or convicted of



Lee M. Thomas

perjury. Thomas, a career bureaucrat, has been assistant administrator in charge of the "superfund" toxic waste programme.

Thomas, whose appointment has to be ratified by the Senate, is a political independent who seems to be acceptable to environmental groups. But with the President under strong pressure to find budget cuts, EPA must be a possible target. Richard Ayers, senior staff attorney with the Natural Resources Defense Council, wonders whether Thomas will carry the political weight to defend the agency, whose operating budget, at \$1,200 million, is still less than it was in 1980 in nominal dollars. Earlier this year, Thomas told the House of Representatives that a proposed budget increase for the superfund programme, tied to a new timetable, would actually slow down the superfund work.

Tim Beardsley

Natural environment

UK council counts pennies

THE British Natural Environment Research Council (NERC) acknowledged last week that it can no longer adequately support those who look to it for research funds. According to Mr Hugh Fish, the new chairman, NERC has been squeezed by the general decline of government support and by the unwillingness of government departments to commission research. Speaking at the launch of NERC's annual report, Mr Fish said the council would have to become more selective, and promised a "corporate plan" early in 1985 that might identify areas of research to be temporarily or permanently abandoned.

Anticipating the forthcoming corporate plan, Mr Fish thought there would be a bright future for deep seismic sounding, where grant applications are buoyant. By contrast, remote sensing has been disappointing. Dr John Bowman, NERC's secretary, said last week that the process of peer review may not be the most effective, but that NERC's activity in this field may be more pointed because of plans to work more closely with the Science and Engineering Research Council.

NERC's constitutional difficulty is the large proportion of its budget committed to its own institutions, which took two-thirds of what the council had to spend last year. By comparison, support for academic research last year amounted only to 17 per cent of what NERC spent. Mr Fish would like to see this proportion doubled, but he did not say by when.

As things are, in its support of terrestrial and aquatic life sciences, and of environmental studies in the physical sciences, NERC was able to meet less than a quarter by value of the grants applied for. Its report for last year records regret at not being able to support work in remote sensing of the sea surface, vegetation and soil hydrology and the development of geological ion probes. In short, NERC cuts the figure of a harassed housewife on a restricted house-keeping budget.

So why, for example, spend £10 million on support of the British Antarctic Survey? Mr Fish is glad that the British government has given this field priority and would like "similar treatment for other areas".

In one respect, however, NERC has been able to break new ground. The council has taken steps to market its services as a research organization willing to take on commissions, and has set up an office in Brussels to ensure that its constituent laboratories derive as much benefit as possible from the European Commission's budget. The report even offers the services of its Brussels office to British universities that might wish to make use of them — but at a fee.

Philip Campbell

US shuttle delay

More trouble with loose tiles

Washington

NEW technical problems with the heat insulation tiles of the space shuttle orbiter *Challenger* have forced the National Aeronautics and Space Administration (NASA) to postpone several scientific missions scheduled for next year. Worst-hit will be the Spacelab Earth Observation Mission (EOM-1), which will not now fly in July next year as had been hoped but will probably be merged with EOM-2 and flown in September 1986. EOM-1 consists largely of instruments that could not be used effectively on the November 1983 Spacelab 1 mission because of the delays to that flight. The Spacelab 2 and Spacelab 3 missions will also both be delayed by several months.

The latest delays will certainly increase the cost of keeping together the scientific groups in both Europe and Japan that have contributed experiments. The proposed rescheduling of EOM-1 was one of the topics discussed last week by Professor Reimar Lüst, director general of the European Space Agency, and NASA administrator James Beggs. The delays will also further tarnish NASA's image with the Department of Defense (DoD): a classified payload that had been scheduled for launch in *Challenger* last month has been shifted to January, when *Discovery* will be used instead. NASA is obliged to give DoD first priority on shuttle flights, with commercial payloads ranking second and scientific missions last.

The problems with *Challenger's* heat insulation tiles have been caused by an unforeseen chemical reaction between a water-resistant spray used to protect the tiles from rain before launch and the adhesive holding the tiles to the orbiter's aluminium skin. New thicker tiles are now being fitted in critical areas using new techniques while other tiles are being replaced by an insulator fitted in blanket form. The modifications are likely to take at least a month, and *Discovery* will probably need similar attention next year.

NASA officials stressed last week that if, as seems likely, EOM-1 is delayed until the autumn of 1986, there will be some scientific benefits to compensate for the inconvenience and expense of waiting for an extra 14 months. Because the combined mission would not have to accommodate any other free-flying payloads, observation time should be close to the full 6 days' effective duration of the mission, in contrast with the 4 days of work that could be expected from a July 1985 flight. And, one official pointed out with engaging frankness, a combined mission will have a higher priority ranking than either would separately, and so is less likely to be affected by any future technical difficulties that cause flights to be rescheduled.

Although no final decisions have been

taken on which experiments would be used in a combined flight, there is no major problem of incompatibility of candidate instruments and it should not be necessary to drop any experiments. The major constraint will be the amount of time the orbiter must spend in different attitudes for different observations. The experiments that had been scheduled for a 1985 EOM-1 flight include several solar radiometers and spectrometers for which the shuttle cargo bay has to face directly towards the Sun, as well as an ultraviolet telescope to be pointed into deep space and a German/European Space Agency metric camera for Earth observation, both of which flew in Spacelab-1 but were not able to obtain all the desired data. The September launch date now being canvassed would allow growing-season observations of vegetation in Europe and is thought to be ideal for the metric camera. The add-on experiments that had been provisionally assigned to EOM-2 will observe the limb — the horizon as seen from space — and also require the cargo bay to be facing Earth.

The EOM-2 instruments are designed to

Royal Society

Dependant bites benefactor's hand

THE Royal Society threw its weight last week into the argument about the conduct of research policy in Britain, but in the measured language people expect of it. Like Sir Keith Joseph, the British government's Secretary of State for Education and Science, the Royal Society's president until next year, Sir Andrew Huxley, said last week (in his annual address) that he had been moved to worry about the condition of British academic science by a report on the proportion of good research applications being turned down by the research councils. The report, commissioned by the British Science and Engineering Research Council (SERC) was prepared by Sir Jack Lewis (Cambridge).

Everyone has his own idea of what is wrong. To Sir Andrew Huxley last week, it seemed important that support for basic science in Britain "is being milked" to support applied science which, in his opinion, should be the responsibility of the Department of Trade and Industry.

On CERN, the European high-energy physics laboratory at Geneva, and the question of whether Britain should continue as a member, Sir Andrew said that he could not understand why there is such a fuss when the cost of membership has fallen from 30 per cent of SERC's budget in 1978 to just over 20 per cent now. Referring in passing, and apparently dismissively, to the British government's recent proposal that there should be a European academy

look at the effects of solar variability on the stratosphere, and in particular ozone concentration. One of these add-on experiments, called ATMOS (Atmosphere Trace Molecule Spectroscopy), will benefit from being in a 57° inclination orbit rather than the 28.5° orbit that had been planned for EOM-2, and the combined flight will offer the opportunity to compare the performance of instruments that would not otherwise fly together. Time competition from the solar observation experiments should not be a major difficulty, as all the solar observations can be completed in two 12-hour sessions.

There will, however, be real scientific losses for experiments that might have flown on both EOM flights, such as a Belgian grille spectrometer experiment that will be used for comparisons with results from ATMOS. Other instruments might have been flown in EOM-2 after modifications based on experience with EOM-1. Dr Doug Torr of Utah State University, who works on the Imaging Spectrometric Observatory (ISO), says that experiment was to have been used in different modes on the two flights and will have to be reconfigured. ISO, which obtains spectra of atmospheric airglow, could provide data on "shuttle glow" relevant to designing the planned US space station. **Tim Beardsley**

named after Leonardo da Vinci, Huxley said that the benefit of that "would be trifling in comparison with the injury that would be inflicted by a body-blow such as Britain's withdrawal from CERN". Procedurally, Huxley does not want to see the British government pay for continued membership out of some other pocket than the science budget.

Perhaps the Royal Society's most timely disagreement with the British government was over the question of student maintenance grants, which are to be made overall less generous so as to increase the funds for academic research by some £24 million a year. Huxley said last week that he welcomed the result but "I deeply regret that these funds have been secured at the expense of students in higher education".

As a reflection of its discontent, the Royal Society seems to be planning a more deliberate, but still modest, increase of its support for studies of the present state of affairs. An inquiry into the "health of basic science" (which will concentrate on research, and the decline thereof, into solid state physics and chemistry and genetics) is being supported by two full-time research assistants. The society is also planning to recruit three professional people, and supporting staff, to keep an eye on the development of science policy in Britain. To ensure independence, the society will not use government money for the task.

John Maddox

Problems of defining length

SIR — Asher Peres¹ discusses three aspects of length measurement: the definition of the SI unit of length, the realization of that unit, and the use of the unit to perform a measurement, and touches on problems that we have already discussed^{2,3}.

The relevant postulates of Einstein's relativity theory have been established experimentally with an accuracy well beyond that of the present best methods of realizing the SI metre; particularly so for Michelson-Morley experiments⁴. The nature of length has not been detectably changed by the new definition, nor has the size of the SI unit. The Conference Générale des Poids et Mesures (CGPM) has not recommended any changes in the laws required for scaling up or down from the unit length in order to make length measurements, neither is one obliged to use light for this purpose. The unit is merely a reproducible scalar representation of the physical quantity against which other lengths may be compared. The only change is the manner in which the new definition is specified and realized. The velocity of light is not asserted to be constant, but if it is changing, then the size of at least one of the SI units will be changing also — relative to some absolute units to which we do not yet have access (the units of *gedanken* metrology). Thus for cosmological experiments, where the constancy of the unit is required over distances and times, comparable with the size and age of the universe, respectively, neither the SI units nor the natural units (e.g. where $e = m_e = c = 1$) can necessarily be relied on. The SI units are of a pragmatic nature, and are designed to provide the most invariant units that can be achieved with the science and technology of the day: if the present methods become detectably inadequate they will be replaced by something better.

The definition of a unit tells us little about the nature of the physical quantity concerned, and we need other instructions in order to know how to measure a physical quantity. The definition of the kilogram, for example, is clearly not intended to define the concept of mass, or to instruct on how masses should be intercompared. Similarly the definition of the metre is not intended to be a definition of what we mean by length, or of how lengths must be measured. The CGPM has redefined the SI unit of length, but not the meaning of length itself.

The CGPM provides a definition of the unit⁵ which gives sufficient information for the great majority of users. The definition is accompanied by a *mise en pratique* which contains information and recommendations concerning the manner by which the unit should be realized if the full accuracy is required. The onus is on any metrologist who chooses to use a different method to demonstrate that the unit so realized is of the same magnitude as that

produced by the recommended methods — moreover, it is stated that proper use must be made of the state of knowledge of the day in applying corrections for known effects (whether electromagnetic, quantum-electrodynamic or relativistic effects, and so on). Professor Peres is correct in stating that although the new definition might be thought to require two synchronized clocks, the practicality is that the round trip time is measured and divided by two. This is indeed the current practice in both interferometry and time of flight measurements (such as lunar ranging) and is implicit in the *mise en pratique*.

The CGPM recommendations are the result of extensive consultations with scientists and technologists throughout the world, but they cannot anticipate future discoveries other than by extrapolating existing trends. For example, if there were found to be small, but significant, anisotropy in the velocity of light (that is some preferred axis in space) then this property would either be incorporated in the definition itself or in the *mise en pratique*. At present we know that the new definition can be realized with a considerably greater accuracy than could be achieved with the former krypton-86

realization of the metre.

On the question of the use of the unit, Professor Peres uses light to measure the circumference of the Earth and so it follows that it must be in some form of waveguide in order to circumnavigate the Earth. In such a case it would, of course, be necessary to take account of a number of effects and to make the appropriate corrections, including that for the rotation of the Earth.

Relativistic effects can be encountered with measurements of mass, length, time, in electromagnetism and elsewhere, however their base units are defined. As accuracies improve, it will be necessary for more and more people to take these and other sophisticated effects into account before interpreting the results of measurements. It is certainly important to ensure that the units of experimental and theoretical sciences remain conceptually consistent whenever changes are made.

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English at risk

SIR — Your invitation (*Nature* **306**, 134; 1983) to readers for comments on the abuse of language seems to have evoked little response. May I reopen the issue?

Idso and Brazel (*Nature* **312**, 51; 1984) write: "Inclusion of this latter effect in a simple model of watershed runoff applied to 12 drainage basins in Arizona..." Various points made in the letter, and the North American addresses of the authors, suggest that by watershed they mean a catchment area.

So what distinction is being made in the quoted passage? Or is it just what Fowler and Fowler called "elegant variation"? I have already pointed out (*Nature* **307**, 312; 1984) that the meaning of watershed is so uncertain that it is unsuitable for use in the mixed company of an international readership.

A new verbal abuse is that the distinction between credibility and credence is in danger. A statement has credibility, credence is something given to it by a human mind. *Nature* is concerned with the written rather than the spoken word. May I however grumble about the error, often heard in radio science programmes, of pronouncing dissect as if were spelt disect. The word means "to cut apart" not "to cut in two", and one of the few almost unbroken rules of English spelling is that a double consonant shortens the preceding vowel.

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Exosat

SIR — Your news item "Britain in space — strong links with Europe urged" (8 November, p.92) refers to "some recent failures of instruments built for other people's satellites (such as some of the detectors for the Exosat satellite)". We should like to point out that the Exosat detectors which have failed were not built by a British space research group but by industrial contractors selected by the European Space Agency. The early development of the prototype detectors was carried out in this laboratory, but, contrary to previous practice, none of the flight hardware was built here. This point seems to have escaped many people's attention.

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Not our drugs

SIR — I write to draw attention to a substantial mistake in your leading article "What price drugs?" (15 November p. 184)).

The drugs Valium and Mogadon are produced by Roche Products Limited and not by Ciba-Geigy Pharmaceuticals; we are in no way involved with their production or marketing.
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Science and the philosophers

from George Gale

To scientists, the philosophy of science seems an irrelevance, as does the empirical practice of science to philosophers, preoccupied as they are with the logical consistency of their methods. The gulf between the philosophy of science, which has its roots in the growth of positivism in the late nineteenth century, impoverishes both. But there is now hope that the gulf will be bridged by the evolution of philosophy into theory of science.

THE activities of the philosopher are simply irrelevant to my scientific life. This view, or one very similar to it, is most certainly held by a vast majority of practising scientists in the English-speaking world. It was not always so. Philosophy and the various sciences have long illustrated the intellectual analogy of the relation between parent and offspring, in which each new scientific discipline, in its turn, left its philosophical home in order to embark on its own independent career. Moreover, historical research clearly reveals that philosopher and scientist often cooperate closely during periods of conceptual crisis or revolution in the sciences in question, particularly when the issues involve methodological problems¹. For similar reasons, this pattern is also exhibited during the initial phases of newly developing sciences, as will be seen.

As might be expected, given a relationship of this sort, cooperation between philosopher and scientist lessens during periods of conceptual calm in a science, as the scientist engages himself in the everyday work of the laboratory and classroom. Physicist John Urani, among others, has suggested that present-day physicists' lack of interest in philosophical matters illustrates their contentment with the conceptual structure and methodology of their science as it is.

Yet the present-day scientist's disdainful attitude towards the activities of the philosopher goes far beyond what might be explained as the result of intellectual contentment. Something quite drastic has occurred, something which has severely weakened an interdisciplinary connection stretching back to the Greeks. In what follows, I attempt to uncover some of the elements involved, looking from the philosopher's side of the science-philosophy relationship.

Naturally enough, it is to be expected that analysis of such a fundamental issue would be quite a complicated effort, involving as it must discussions of recent historical developments in both science and philosophy; discussions about the struc-

ture and goals of contemporary higher education; and even discussions about practical features of the internal political structure of both professions. But despite the complexity and difficulty, such an effort clearly needs to be made if we are to understand the genesis of such a radically intensified view as that held by contemporary scientists in the English-speaking community. It is hoped that the discussion will serve as an initial effort to begin examination of this enormously significant topic.

Marriage and divorce

Philosophy and science emerged together, mixed and intermingled inchoately in the first attempts by classical Greek thinkers to understand nature. But even though they always remained linked in Classical thought, it did not take long for appropriate roles to be sorted out. Thus, already by the time of Aristotle, certain very definite demarcations had become apparent between the two disciplines. It is fascinating that, even today, when one reads any particular treatise of Aristotle's, it remains quite easy to see when he is thinking as a philosopher of science rather than as a practising scientist. Indeed, many topics originally developed by Aristotle continue to hold interest today. Thus it is that contemporary philosophers retain strong interest in both the logic of scientific thought and the structure of scientific method, two primary targets that were originally identified by Aristotle and his successors.

Strong interaction between philosophy and science is not, however, limited to the period of classical Greece. Right from the initial awakening in Copernicus, Kepler and Galileo, modern science and modern philosophy emerged together, to interact with one another in the production of a mature synthesis, a true world view, through the efforts of great philosopher/scientists such as Descartes, Leibniz and Newton². It is no accident that Newton thought of himself as a natural philosopher, that is, a philosopher of nature.

These examples demonstrate the closeness of the relationship between science and philosophy during the conceptual revolutions of the formative period of modern science. Scientists and philosophers both believed strongly that the two disciplines are inextricably linked in the general structure of human knowledge itself. For this reason, they believed that interaction was not only beneficial but necessary to the ultimate success of human inquiry into nature. Indeed, Descartes, Leibniz and Newton believed so strongly in this connection that they took it for granted and worked actively in both disciplines. Later, even after scientific professionalization had begun to decrease somewhat the number of scientist-philosophers, philosophers and philosophy itself remained in close and beneficial communication with the sciences. Immanuel Kant, the great eighteenth century German philosopher, in fact regarded providing the philosophical foundations for classical physics to be his most significant task³. His efforts endured for nearly a century as hallmarks of the science-philosophy connection.

After Kant, the eras closer to our own time continued to exhibit the connection between science and philosophy. In biology, Darwin and the philosopher Whewell argued long, hard and successfully in critical analysis of the conceptual and methodological content of the revolutionary theory of evolution. Later, when early twentieth century physics experienced what can only be called revolutionary upheavals, as the theory of relativity and quantum physics overthrew the classical world view, the classical philosophy developed by Descartes, Leibniz, Newton and Kant was overthrown as well. Indeed, it can be argued convincingly that the philosophical consequences of these two new physical theories were at least as important as the theories themselves.

Such would most certainly be the view of many of the participants in the revolution; Bohr, Einstein, Planck, Heisenberg, DeBroglie among many others⁴. Thus, it

would seem that at least in that most fundamental of the sciences, physics, communication and interaction between philosophy and science continued unabated at least until late in the third decade of this present century. (Indeed, a small community of scientist-philosopher collaborators continues to work in both quantum mechanics and relativity theory, an obvious exception to the general situation.)

In the face of such a vigorous historical tradition, today's perceived irrelevance of philosophy to science stands out starkly. One can only ask, "What in the world happened?" Initial answers to this question must focus first upon internal topical developments in recent philosophy.

Reforming science

The ultimate irony of the present divergence between scientists and the philosophers is that its roots lie in the efforts of a philosopher-scientist to bring about a philosophical reform in physics. Ernest Mach, the philosopher-scientist in question, believed that physics — and the natural sciences in general — had become seriously infected by the post-Kantian metaphysical idealism that dominated the faculties of major German universities. In particular, he argued that the easy acceptance of hypothetical concepts, concepts which typically referred to unobservable theoretical entities such as energy, force and absolute space and time, had proliferated to the point where physics was populated by an incredible metaphysical bestiary.

Mach sought to reform physics entirely, to chop, prune and pare its theoretical structure until only what was "positive science" remained. From this stated desire came the general rubric for the ensuing reform. Following the notions originated by Comte, Mach and his successors took up the movement called "positivism"; and while there are other important reasons behind the present disaffection between science and the philosophers, those reasons which are substantive, and rely upon topical disciplinary content, lie mostly in the beliefs of positivism and their effect upon several generations of philosophers in the English-speaking world.

Positivism has three tenets, each of which entered the doctrine at a different time, under the aegis of a different individual⁵. Mach, in 1886, provided the "empiricist criterion of meaning"⁶. Poincaré (1902) suggested the "conventional view of mathematics"⁷. Whitehead and Russell (1910–1913) developed "the axiomatic system for symbolic logic"⁸. And finally, in 1923, Rudolf Carnap introduced a complicated synthesis of the three elements in what can be clearly recognized as what we today call "positivism"⁹. (The movement is also variously called "logical positivism" and "logical empiricism" in obvious reference to alternative emphases on various of the

major principles.)

Each of the three principles contributes something distinct to the positivist theory about scientific theory. Mach's empiricist criterion of meaning represents his attempt to eliminate reference to unobservable entities from science. Roughly stated, the criterion disallows from acceptance into legitimate science all terms that do not have an empirically observable entity, or event, as a referent. Since, for example, according to Mach, the term "absolute space" does not name an object or event which is observable in the physical/empirical world, absolute space must not be allowed as a concept in legitimate science. In this fashion, both observational terms and observation itself became the essence of science. Bridgman's operationalism and Popper's falsificationism are obvious inheritors of this tradition.

Unfortunately, one initial logical consequence of Mach's empiricism was the required elimination of mathematical terms and operations from properly cleansed and thus legitimized theories. This grotesque result follows from the fact that purely mathematical concepts have no observable referents; for example, "plus", "sum" and "differential" are not the proper names of any empirically observable physical object or event. Further, since the empiricist criterion of meaning also laid down the rule for which sentences could qualify as being true or false, all mathematical sentences automatically became meaningless. After all, if theoretical terms are meaningful only if they have observable referents, then all mathematical terms are meaningless precisely because they lack such referents. It follows directly that mathematical sentences, which contain only meaningless terms, must be themselves meaningless, and cannot even be candidates for truth or falsity.

Maths and truth

Recognizing the unsatisfactoriness of this bizarre result, Henri Poincaré found a way minimally to preserve mathematics and yet hold to the empiricist criterion at the same time. His proposal, stated roughly, was that mathematics, while it is not strictly speaking empirical, is, even so, associated with the conventional activities of scientists. That is, even if mathematical statements cannot be candidates for truth or falsity, at least they are not meaningless, since they capture meaning from the conventional practices of theoretical scientists. Moreover, according to Poincaré, if necessary, mathematical sentences are ultimately eliminable from science, since their substantive content is reducible to strings of sentences containing only empirically meaningful observational terms. On this view, it is clear that the formalisms of scientific theory itself are, strictly speaking, redundant, since they represent only the conventional way scientists speak about what, in the end, reduces to strings

of observation terms. After Poincaré, positivism lacked only a suitably precise language in which to carry out the proposed reform of physics. That language was provided by the achievement of an axiomatic system for symbolic logic.

Philosophical concern to impose logical methods on scientific thinking is an ancient and honourable endeavour. It was Aristotle himself who laid it down that deductive logical structure is a necessary condition for any discipline to call itself "scientific". This demand was scarcely relaxed during the development of the modern philosophy and science, as Descartes and Leibniz clearly evidence. But what had always led to failure in previous attempts to make science logical was the lack of a sufficiently rich, powerful and precise logical apparatus. Deductive syllogisms produced from the syntax and semantics of everyday speech just could not do the trick. Whitehead and Russell's axiomatic system for symbolic logic, the logic of quantifiers and predicates with identity, made available for the first time a language which seemed to offer the potential to capture significant aspects of scientific thoughts. Unfortunately, as was later revealed, logicistic efforts cause more problems than they solved. But, at first, optimism reigned.

Rudolf Carnap produced the first mature interpretation of logical positivism, a synthesis in which the three original principles were the foundation. According to this view, legitimate scientific theories were to be constructed along the following lines. At the foundation level, terms were to be introduced in strict association with observation, and put into definitions stated in the predicate calculus. "Space", for example, would be associated with "measurements made with a ruler", "time" with "measurements made with a clock" and so on.

It was even hoped that complex predicates such as "soluble" and "homeostatic" would succumb to this analysis. Once these terms were safely stated in the new symbolic logical system, they would become amenable to manipulation by logical operations and accepted mathematical functions. Theoretical terms would emerge from these operations. "Velocity", for example, could be introduced as the "dividend" of 'space divided by time', and "acceleration" could then appear as a further dividend. In this stepwise process, all theoretical terms could be examined, analysed and ultimately rejected if they failed logical derivation of this sort.

Carnap's programme was quickly hailed as the first successful candidate to fulfill the philosophers' millennial-old desire to apply their distinctive technique of deductive logical analysis directly to scientific thinking. But terrible problems immediately surfaced. For the most part, these were intrinsic to the positivist programme itself.

Cosmologist Brandon Carter has

recently noted one particularly prickly issue. Even given Poincaré's conventionalist interpretation, accepting the empiricist criterion of meaning causes problems for those whose scientific activities most intimately involve mathematics, the theoretical physicists. In the first place, Carter notes, theoretical physicists are most uncomfortable with the notion that mathematical results might be meaningful (in a certain limited sense), but could not be candidates for falsity or truth. Moreover, he believes that most theoretical physicists would in nearly all cases feel much less confidence in assigning truth or falsity to sentences employing empirical observation terms than to mathematical sentences. As Carter remarks, he is much more comfortable trying to decide whether a mathematical sentence is true than he would be faced with such a decision regarding a statement about the physical world.

The response of many philosophers to Carter's view would be revealing. "Carter does not understand the nature either of truth or of mathematical systems", they would claim. Many would argue that his view is naive, indicating a need of education in modern philosophical theories. Such a response is clear evidence of the breakdown in communication between philosophy and the scientists. Regardless of the correctness of either side, it is clear that neither is attending properly to the other.

Reform

One aspect of this situation is especially ironic. A philosophical reform movement instituted by a theoretical physicist specifically to cleanse his own discipline philosophically, has succeeded in alienating a sizeable proportion of the practitioners of that very craft from an interest in either philosophers or philosophical matters.

Several further points must be mentioned. First, although some attempts have been made to extend positivist programmes beyond physics (one might think here of behaviourist psychology, which began life as a discipline specifically constructed according to the guidelines of positivism), for the most part, positivist philosophers have focused on physics. Such a limited perspective on science has of course limited the range of relevance of recent philosophy of science. Given the developments in many sciences over the past sixty years, this restricted focus has isolated philosophers from a vast number of new and/or improved sciences.

A second problem involves the positivist language itself. Modern symbolic logic has itself matured into a discipline with a highly developed system of techniques and processes. In order even cursorily to follow relevant discussions by modern logically-minded philosophers, scientists must become fluent in the symbolic language. Since they cannot be expected to do so, it becomes incumbent upon philosophers

themselves to initiate and maintain communication and interaction. But there is little evidence that this has happened.

In fact, it appears that interest in logical analysis and other formal questions has quite isolated a sizeable segment of the philosophical community, keeping them from being concerned with issues that concern their scientific colleagues. As physicist James Cushing has noted, this sort of interest has introduced issues into the philosophy of science that do not occur in actual science. Thus, scientific questions which might once have been of interest no longer attract philosophers, in part because they lie outside current philosophical practice.

Accordingly, if a scientific issue does not involve formalizable questions, then it does not concern logic-oriented philosophers. One example is the stance of philosophers with regard to the anthropic principle, a significant topic in cosmology for almost fifteen years. This principle raises both methodological and substantive questions in that it claims that the existence of human beings is not a neutral fact in the Universe but that our existence, by itself alone, has implications for what may be observed — and theorized — in cosmology¹⁰. Issues such as this should be of obvious interest both to philosophers generally engaged in traditional enquiry about deep human concerns and also to those whose focus is more narrowly on the analysis of scientific methodology. Yet little interest has surface about the first concern and essentially none about the second.

Indeed, when I recently discussed the topic in print, almost all the letters and comments I received from philosophers were abusive¹¹. The comment "You have set philosophy back to the time of Copernicus" is typical. I was asked why I "even bothered to interest myself in such an issue". My only reply, then as now, was to note that any scientific development as significant as the use of anthropic arguments represents not only a *legitimate* topic for philosophers, but also a topic that is important, given that so many excellent scientists occupy themselves with it. Not to take a professional interest represents, again, the growing isolation of philosophy from science.

One final factor is perhaps the most important, given the growing professionalization of philosophy and especially of the philosophy of science. As it turns out, the application of the logical calculus to scientific thinking contains its own insidious problems. Since in any general case, interpretive use of a theoretical system imparts into the resulting interpretation flaws endemic to the theoretical system itself, it is not odd that this kind of interpretive use of the logical calculus has often revealed weaknesses not of scientific thinking, but of logical theory. Thus, in attempts to interpret the methods by which scientists set about confirming,

verifying or accepting hypotheses, formal problems intrinsic to the logic of the conditional "if, then" operation, which must be used in modelling scientific prediction, soon turned philosophers away from positive accounts of verification through prediction. Instead, falsification, *à la* Sir Karl Popper, was proffered as the proper legitimate mode of assessing scientific theory, for no other reason than that its logic is much cleaner than that of prediction¹². According to the falsificationist model, scientists' ultimate task is to falsify and discard theories rather than to confirm and accept them. Regardless of whatever other virtues there might be in the falsification programme, and Popper's programme especially, such a conception of the ultimate scientific task is bound to seem odd to thinkers who believe their scientific lives to involve a search for truths about nature.

But even more important is what has happened to professional philosophers as a result of the intricacies of the logical calculus they have adopted as an instrument. In effect, philosophers have been enticed away from the problems of interpreting science in order to focus on problems internal to the means of interpretation rather than the substance. A survey of the philosophical literature during the 1940s, 1950s and 1960s reveals a proliferation of articles devoted to complicated and technical questions of a logical nature which, although derivatively related to the analysis of science, are most conspicuous for their primary concern for the details of the logical calculus, not the details of the science. These almost arcane discussions, verging on the scholastic, could have interested only the smallest number of practising scientists.

Coinciding with this inward-turning of perspective, academic philosophy in both Britain and the United States experienced the greatest period of growth in its history. Growth of philosophy faculties entailed expansion of graduate student ranks, which naturally made it necessary to find tasks on which to engage the new philosophy candidates. Happily enough, the internal troubles of the logical model for science were severe enough to absorb even this new flood of workers. So the logical positivist theory became doctrine, with a vast new influx of defenders and practitioners. Among philosophers who now professionally interest themselves in science, a graduate student background in positivism predominates. And although it would be unfair to say that the formalistic issues of the logical positivist programme still represent the doctrinal concern of philosophy of science in general, there persists a major thrust in that direction. Many philosophers would agree with the leading protagonist of this effort who recently told me that scientific thought is philosophically interesting only to the extent that it is formalizable.

The upshot of all this is that, because of

its substantive content, the past two generations of philosophical concern with scientific thought have increasingly driven the philosophers away from their traditional interaction with science in general, have restricted philosophical focus to a narrow range of physical theorizing and, finally, have turned the gaze of philosophers away from science itself and towards the internal deficiencies of the means of analysis, symbolic logic. This movement has had disastrous effects on communication between philosopher and scientist. Unfortunately, aiding and abetting these internal topical developments in philosophy, there has been a series of developments in the academic politics of both philosophy and higher education in general which has exacerbated the crisis of divergence.

The wider context

Accompanying the growth of philosophy faculties in both Britain and the United States was a concomitant growth in professionalization, especially noticeable among the ranks of the philosophers of science. Indeed, it is not outrageously unfair to say that philosophy of science came into existence as a profession, separate from philosophy itself, during this period.

To a certain extent, this professionalization of the philosophy of science required the emergence of an identifiable set of commitments and beliefs that could function as professionally unifying tenets. Logical positivism satisfied this function. Although not all philosophers of science identified themselves as positivists, positivism provided the standard against which to define themselves, positively or negatively. And the positivist tenor of the times was inescapable, even for those who foreswore the doctrine itself.

All the institutional hallmarks of professionalism accompanied the doctrinal unification. Membership in professional societies grew and, in turn, the various activities typical of professional societies strengthened the profession. Conventions, professional colloquia, the publication of journals and the like cemented the membership together, providing a sense of purpose and, in general, reaffirming the substantive movement of the times. The ultimate institutional outcome of this professionalization showed itself in two ways. "Philosophy of science" positions were provided in nearly all sufficiently large departments of philosophy while departments devoted solely to philosophy (and latterly, history) of science were instituted at a number of important institutions. But contrary to what might have been expected, institutionalization of the profession raised problems for the philosophy/science connection.

To keep an academic department healthy, especially one as specialized as in philosophy and history of science, a certain mass is necessary to ensure direct interac-

tion and cross-fertilization. When a department is too small, healthy diversity of opinion, tolerance for other views and intradepartmental learning, seems never to arise. In short, departments fail to grow in perspective and knowledge, and one particular viewpoint tends to dominate.

Problems of critical mass have always plagued the specialized philosophy of science groups in Britain, which could certainly explain the apparently monolithic doctrinal nature of British philosophy of science. With budgets short, the prospects for improvement are not bright.

A likely consequence of this unhealthy situation is stagnation in philosophical research, and an attendant inability to turn away from internal institutional problems towards interaction with scientists. With the stance of philosophy already directing energy from direct involvement with genuine scientific practice, the ultimate results are likely to be disastrous.

A second quite different problem, concerns the internal politics of philosophy departments. In the recent past, most Anglo-American philosophy departments have taken on an analytic cast, with "Wittgensteinian" departments (and there are many) representing the most extreme cases. Philosophers of science, even where special philosophy of science sections are integrated into the general departmental structure, are always in the minority. They are thus subject to any consequence that may derive from the way in which the nature of their professional interest conflicts with the analytic nature of the greater department. To put it bluntly, conflict appears to be inevitable.

Contemporary analytic philosophy often expresses a strong mistrust, indeed hostility, towards the empirical world and to those who even give the appearance of dealing with it. Views such as this are not new, but are familiar from the time of Plato. But in its modern guise, analytic philosophy has always emphasized the limitation of its interest to the conceptual aspects of human experience, eschewing concern for the empirical world of nature.

The political repercussions of this stance were not long delayed in the newly-professionalized philosophy of science. Insofar as a person's work exhibited elements that might be construed to be "empirical", his work was thereby held by the majority to be "non-philosophical". Moreover, contact with real practising scientists was at least questionable from the point of view of authentic philosophy. Since departmental political power typically rests in the hands of others than philosophers of science, conflicts such as this raise problems not only for philosophers of science in general but, particularly for those who might have interests in close engagements with individual scientists or empirical scientific practice, both of which require of philosophers extensive immersion in the empirical content of the target scientific disciplines.

The consequences of these political problems are all too familiar. In one case, for example, hostility from a strongly analytic department has recently forced a well-known empirically-minded philosopher of science to change institutions. In another case, a philosopher in a large British philosophy/philosophy of science department has been the target of hostility from the majority of other philosophers, simply because he has been congenial to members of the philosophy of science group. One need not even ask about the status, in this department, of the philosophers of science themselves.

In other cases, philosophers of science who tackle empirical problems, who appear on programmes of scientific meetings or who publish in scientific journals have difficulty convincing their departments, or the various academic review boards, that their work is proper "professional" philosophy. When competition for salary, tenure and recognition is intense, such problems are of great practical importance. And even if these are isolated cases, and not withstanding this intradepartmental hostility in every kind of department, philosophy or other, it is clear from the style and tone of the cases which have arisen that there is specific animus mitigating strongly against contemporary philosophers of science who would initiate the first difficult moves to re-establish their part of the traditional link with the community of scientists.

One idiosyncratic feature of the United States is that component of American mythology, which has it that the philosopher is the quintessential theorist, the paradigm of the deep thinker whose head-in-the-clouds activities render him irrelevant to the practical world. Given this longstanding attitude, it is mystifying that US universities even bother to maintain departments of philosophy. But in the recent vigorous tailoring of US university education to the requirements of professions and the workplace, perceptions of the relevance of philosophy have further diminished as courses have been dropped, programmes curtailed and the number of majors have plummeted.

Naturally enough, philosophy of science is tarred with exactly the same brush, the internal divisions in philosophy departments notwithstanding. The academic community's growing perception of the irrelevance of the philosopher of science *qua* philosopher, when coupled with the restrictive concerns of the discipline itself, make it almost inevitable that contemporary science students in the United States will not only develop in an environment where philosophy is taken to be irrelevant, but will have only a slim chance of encountering those who might demonstrate otherwise. So stands the situation today. Fortunately, there are some signs that, at least in some areas, further divergence between philosophy and science is not unavoidable.

A brighter future?

Several recent developments suggest at least a hope of some improvement in the relationship between science and philosophy which involve, on the one hand, some of the newer sciences as well as theoretical biology and, on the other, doctrinal changes in philosophy of science itself.

Clark Glymour has recently noted that newly emerging sciences tend to provide considerably more opportunity for relationships between philosophers and scientists. He pointed to computer science and, in particular, artificial intelligence, where there has been close collaboration between scientists and philosophers. Interestingly enough, this relationship extends even into substantive matters; one recent expert system, a medical diagnosis routine, was designed from the outset to embody a falsification testing strategy. It will be more than slightly ironic if the logical formalization programme then turns out to provide an acceptable basis for artificial scientific intelligence when it has not been accepted as a basis for the more natural versions of scientific intelligence.

Contemporary biology is another field of increasing interaction between scientists and philosophers. Whereas twenty-five years ago, respectable biologists would have had little or no interest in philosophical issues, contemporary biologists and philosophers are engaged in a surprisingly vigorous interaction with one another. Marjorie Grene, whose philosophical encounters with biology bear witness to the vast changes during this long period, believes that two factors are involved. First, biologists themselves have suffered such sweeping, indeed revolutionary, changes in both concepts and methodology that they are necessarily much more open to philosophical concerns. Moreover, perhaps more important, there is now a generation of biologically well-trained philosophers, whose strong grounding in the empirical details of the science commands the respect of biologists. It is interesting that, as Grene and Glymour note, many of those belonging to the vigorous core of contemporary philosophy of biology were partly trained in Harvard's interdisciplinary programme.

Others have noted that the philosophy of biology has never developed a strong doctrinal allegiance with logical positivism and, for this reason, has not been isolated from its subject as were the more physically oriented philosophers of science.

Radical changes in the substance of philosophy of science also offer some hope. Everywhere in the English-speaking world, but especially in the United States, where the influence of falsificationist

doctrine was never so monopolizing as in Britain, philosophy of science is undergoing a radical shift of methodological orientation, away from the formal and logical towards the empirical and historical. This change has been brought about by the challenges to positivist doctrine embodied in the works of Thomas Kuhn¹³, Norwood Russell Hanson¹⁴ and others.

Some of the issues raised by this revolution go directly to the foundations of the philosophy of science. Thus several philosophers of science, most notably Giere¹⁵, have challenged philosophers and philosophers of science alike with the idea that philosophy of science may itself be an empirical and essentially scientific activity, not the *a priori* conceptual discipline exemplified in traditional departments. Consequentially, there have even been suggestions that this more historical/empirical philosophy of science would be more aptly named "theory of science".

Developments such as these, unfortunately, can only exacerbate the hostility of many traditional philosophers, and that bodes ill, at least in the short run, for the philosophers of science, politically isolated as they already are in their academic department of philosophy. But in the long run, the situation of the philosophers of science may be bettered from a surprising direction — the sciences themselves. A growing emphasis on historical and empirical components in philosophy of science would tend to bring about closer relations between scientists and philosophers, and this for several reasons.

The shift away from the arcana of technical logic towards the language of historical and empirical analysis will facilitate communication between the two disciplines, when scientists most surely will regard philosophers as methodologically and linguistically less alien. Second, it is possible that the subject matter of an historical/empirical philosophy of science would be intrinsically more appealing to scientists; certainly, it is always more interesting to find one's own behaviour and methods described rather than prescribed, as under the earlier positivist regime.

Schism

Finally, as philosophy of science more and more evolves into theory of science, certain very practical effects may compensate for the losses brought about by the schism between the new theorist and his erstwhile base in traditional philosophy. As Henry Frankel has remarked, under such conditions, many elements of the established reward structure in science would be open to the historical/empirical theorist of science. For example, Frankel notes, grants from the US National Science

Foundation might become accessible; Kristen Shrader-Frechette has noted that administrators at US universities are especially appreciative of grant receivers, and doubly so if they are engaged in interdisciplinary activities.

Naturally, it is by no means certain that the possibilities suggested by Giere and others are likely to occur. But if it is considered desirable to establish an essential interaction between two fields which, by both nature and history, should be strongly interacting, fostering the evolution of philosophy of science towards theory of science would be one excellent strategy.

A quite fascinating speculation arises from this possibility. The history of modern science is the history of one field after another moving out from under the sheltering aegis of philosophy, in order to establish independent dominion over a distinguishable empirical territory. Thus emerged cosmology, physics, chemistry, biology and the rest. Were theory of science to emerge as suggested, would it not be none other than the most recent emigre in this long succession? That question raises exciting issues reaching to the foundations of human knowledge, issues that must needs be answered. It is time to begin.

Some of the historical material herein was presented at an American Association for the Advancement of Science Symposium, 21 June 1983, at Logan, Utah. I have benefited immensely from conversations and correspondence with many people, including B. Carter, J. Cushing, H. Frankel, R. Giere, C. Glymour, M. Grene, R. Harré, M. Rees, G. Ross, K. Shrader-Frechette, J. Urani, J. Worrall. The work was supported in part by a Weldon Spring Grant, and the University of Missouri — Kansas City. □

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Similarities among retrovirus proteins

SIR — In recent correspondence to *Nature*, Cianciolo *et al.*¹ reported a region of similarity between the transmembrane protein p15E of murine and feline leukaemia viruses and gp21 of the human T-cell leukaemia viruses types I and II (HTLV-I and -II). We agree that these proteins are indeed similar and draw attention to additional sequence similarities and structural features common to the transmembrane proteins. The transmembrane proteins of Rous sarcoma virus (RSV), bovine leukaemia virus (BLV) and mouse mammary tumour virus (MMTV) are included in the analysis.

Figure 1 shows a schematic comparison of the transmembrane proteins and Fig. 2 depicts three regions of amino acid sequence similarity (regions IA, IB and IC). The following features are present in all of the transmembrane proteins considered:

The amino-terminal domain of the transmembrane proteins has a hydrophobic character and contains region IA. This domain is followed by a strong polar region. The position of the hydrophobic region at the amino terminus of the transmembrane protein is reminiscent of the fusion sequence of other viral transmembrane proteins such as the Sendai and influenza viruses (see ref. 2 for review). For this reason, we speculate that this region of the HTLV envelope may be responsible for the fusion activity observed for HTLV virus³. The spacing of cysteine residues located near the middle of the transmembrane proteins is also conserved. Moreover, these residues occur in a highly conserved amino acid sequence context

REGION	VIRUS	POSITION	SEQUENCE
IA	HTLV-I	315	PVAVVWLVSAALMAGVAGGI
	HTLV-II	372	PJAVHVLVEALAAIGIAGGY
	BLV	317	PVAALTLGLALSGLIGIN
	AKV	439	REPVSLTALALLGLTMMGGI
	FeLV	465	REPLSLTVALMLGLTVGGI
	RSV	423	PTARIFASILAPGVAAQAL
IB	HTLV-I	377	QNRRLDLEWEQGLCKALQEQCRF
	HTLV-II	433	QNRRLDLFWEQGLCKALQEQCCF
	BLV	380	QNRRLDWLYIRLGLCPINEPCCF
	AKV	509	QNRRLDLFLKEGGLCAALKKECCF
	FeLV	535	QNRRLDLFLQEGGLCAALKKECCF
	RSV	479	QNRALDLFLAHGHGEDVAGMCCF
IC	HTLV-I	452	LLLLVILAGPCIL
	HTLV-II	508	LLLLVILFGPCIL
	BLV	460	ALFLFLAEPCLLI
	AKV	590	ILLILLFPGCIL
	FeLV	616	ILLILLFPGCIL
	RSV	564	LLLLVCLIPCLL
	MMTV	630	ALLLVILMIFPIL

Fig. 2 Alignment of three regions of sequence similarity among the transmembrane proteins of the retroviruses mentioned in Fig. 1. Conserved positions are boxed and conservative substitutions underlined. Although MMTV does not show significant similarity in regions IA and IB, it was included because the general structural features described in the text also apply.

(region IB). We postulate that this region is in direct contact with the exterior glycoprotein, and that disulphide bonds are formed between these two cysteine residues and the similarly spaced and conserved cysteine residues close to the middle of the external glycoproteins. Conservation of sequence in this region might be dictated by structural constraints required for protein-protein contact. Potential glycosylation sites are located near the carboxyl end of region IB in most of the viruses considered. Glycosylation can occur at these sites, as the transmembrane proteins of HTLV-I, HTLV-II, BLV and MMTV are glycoproteins.

There is a hydrophobic region near the carboxyl terminus which includes a highly

conserved sequence (region IC). This region has the correct length and position to traverse the cellular membrane. An α -helix is the most favoured configuration of this portion of the transmembrane region^{4,5}. Moreover, this hydrophobic domain terminates with a positively charged amino acid located three residues (except in RSV) carboxyl terminal to a conserved cysteine residue.

A small, highly variable region is coded for by the extreme carboxyl terminus of the envelope gene precursor of retroviruses. This region, designated R, is cleaved from the envelope gene precursor late in maturation in murine viruses⁶.

At present, there is no experimental evidence to discard the involvement of any of the regions of similarity or structural features reported in the immunosuppressive activity demonstrated for murine and feline p15E.

A more extensive analysis of similarities among the p15E as well as other retrovirus proteins is contained in our review of HTLV-I structure³.

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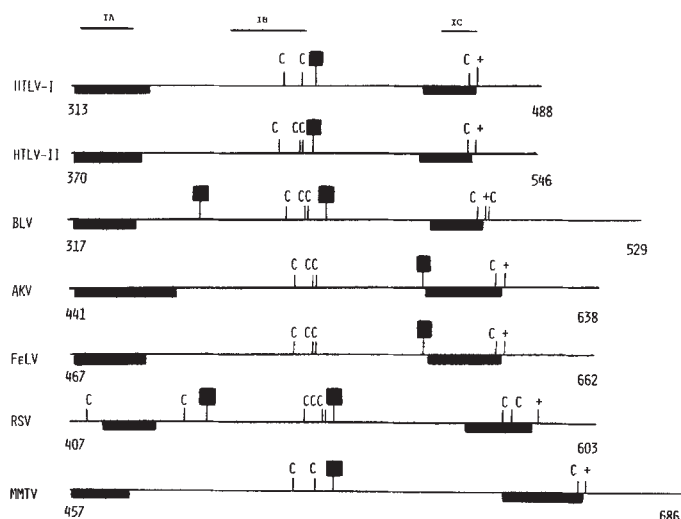


Fig. 1 Schematic comparison of the transmembrane proteins of: human T-cell leukaemia virus types I (ref. 7) and II (ref. 8) (HTLV-I and -II), bovine leukaemia virus⁹ (BLV), AKV⁴, feline leukaemia virus¹⁰ (FeLV), Rous sarcoma virus¹¹ (RSV) and mouse mammary tumour virus¹² (MMTV). The hydrophobicity pattern was obtained using the Intelligenetics PEP program. The hydrophobic regions discussed in the text are indicated by a thick bar beneath the line. Potential glycosylation sites are indicated by a square, cysteine residues by a C and the position of a positively charged amino acid 3' to the transmembrane region is depicted by +. The position of regions IA, IB and IC from Fig. 2 is also shown for HTLV-I.

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Privacy and the peer-review system

There are disturbing signs that the peer-review system for unpublished manuscripts is no longer as secure as it should be. Commercial competition is not the most serious cause of trouble.

MOST complaints about the peer-review system say that it is unjust. But what about the possibility that it has become unworkable? A week ago, a patent attorney wrote on behalf of an author, whose manuscript could not be published in *Nature*, asking whether referees are required to sign a "confidentiality agreement" in advance of being asked to look at authors' manuscripts. His letter went on to allege that the existence of his client's manuscript, and perhaps even its content, had been disclosed to a graduate student at a university in the United States by a member of the faculty, who claimed to have been one of the referees (which is true).

Coincidentally, a colleague of another author (whose manuscript has also been refused) telephoned to say that one of the audience at a recent meeting had chipped in, at the end of a brief formal presentation of the still unpublished work, with a detailed critique based on a sight of the unpublished manuscript, said to be "all over the scientific community". That claim is probably exaggerated; probably the questioner meant "all over my laboratory" (where one of the referees also works). But whatever the truth, the tale is evidence that the peer-review system, whatever its other weaknesses, is not always the confidential means by which colleagues are consulted about the merits of each other's work.

To many people, the cynical and the embittered for obvious reasons, this discovery will not be a surprise. Why, they will say, should scientists who are good enough to help journals (usually without material reward) to decide what to publish also keep their own counsel about the manuscripts that pour endlessly across their desks? And now, when so much of academic science has an obvious commercial value, is it not inevitable that somebody who is actively working in a particular field will make the most of a scientific manuscript sent for review?

There is no denying that access to an unpublished manuscript can often be of great value to a referee. There is a host of fields in which this is the case — the development of semiconductor devices, solid-phase catalysts and photovoltaic devices driven by solar energy, for example. But the competition is especially fierce in the applications of molecular biology to biotechnology where the knowledge of even a short nucleotide sequence of a gene may, for example, enable a competitor to construct the nucleotide probe with whose

help the intact gene may be fished out of a DNA library. That, legend has it, is why people speaking on these topics at meetings in advance of publication tend to show their slides of nucleotide sequences for only very brief intervals — and why there has emerged a group of auditors skilled at memorizing a whole string of nucleotides seen only for a few seconds.

So the incentives for referees to break confidence are considerable. Authors are often painfully aware of the dangers, sometimes to the point of seeming paranoid on the subject. An author's recent claim that a recent piece of published work (which turned out to be mistaken) was being replicated at two other laboratories within a week or so of the submission of the original article was found, on investigation, to be only half-true; the work had been begun before, and was independent. The author had, however, guessed the geographical location of two of the three referees.

These are not the only hazards of the peer-review process, as exemplified by a sad tale from earlier this year. A manuscript reporting part of an incomplete amino acid sequence was in part concerned with what seemed an interesting homology with another amino acid sequence, sent for review to one whose interests include the maintenance of a databank. In the event, the referee spotted a more complete and striking homology than the author had claimed. That information was conveyed to the author together with a tactless suggestion (which was misunderstood) that there should be some formal recognition of what the referee had done. The upshot was that the article was withdrawn (fortunately to be published elsewhere) and the offended author convinced, as he explained, that he "had been robbed of the joy of discovery".

So should referees also take care not to let their intellectual curiosity run riot over papers sent to them for review? Of course not. All journals know of papers that have been enormously improved by the contributions made by referees. Sometimes, authors are ready to acknowledge this, although it must be rare that a referee's contribution is so great that the interpretation of data is entirely transformed. But these, in any case, are not the occasions when authors most fear that their data will be misused before publication.

The problems recently encountered with manuscripts whose content may have

immediate practical value in, for example, biotechnology are much more taxing. The obvious danger is that improper commercial use will be made of data that are still confidential, which is why journals try to use their common sense and not send papers for review to people likely to have a commercial interest in them. But there are two circumstances that make this ideal in general unattainable. First, and quite deliberately, the larger biotechnology companies have made a point of keeping close links with academic science, partly because the step between discovery and practical innovation is so short and partly because it helps in the recruitment of able people. Second, and much more serious, this is now one of many fields of science in which there are very few able academic researchers who do not have links of some kind with some commercial organization, commonly undisclosed.

So has the time come to recognize that the peer-review system has broken down? Again, the answer is flatly no. The interest of patent attorneys suggests that most authors are now keenly aware that the work they seek to report may have commercial value, and that they know what steps must be taken to protect it. Even where research has no immediate application, journals such as this do their best not to send papers for review to groups known to be in competition, or willingly follow an author's request that the referees should not include people working in named laboratories. (Nervous authors would be comforted to know how often referees disqualify themselves on the grounds that they have too direct an interest.)

There is, however, unsystematic evidence, chiefly what seems to be a greater volume of complaint, that some referees are now less than strictly bound by the request that they should deal confidentially not merely with the content of the manuscripts they are sent but even with the fact that they exist. The damage done by such transgressions is intangible but no less worrying on that account. The chief casualty is the civility of academic life. Authors are entitled to expect that their papers will be dealt with privately. Not merely their self-esteem but their public reputation may easily be damaged unfairly by gossip, necessarily incomplete, about the ways in which their latest attempts at publication have been handled. Erring referees should reflect that they are usually authors as well.

John Maddox

Sensory and energy transduction

Light-activated retinal proteins

from Lubert Stryer

THE discovery of bacteriorhodopsin (BR), a light-driven proton pump found in halobacteria, revealed that the photoisomerization of retinal has a significance extending beyond its role in vision in higher organisms¹. Two more light-activated retinal proteins have recently been identified in halobacteria: halorhodopsin (HR), which serves as a chloride ion pump, and sensory rhodopsin (SR), which is the receptor for phototaxis. The genetics, chemistry, conformation, photoreactions and regulation of these halobacterial proteins were explored at a recent workshop*. Discussion of the proteins was enriched by comparing them with rhodopsin, the photoreceptor protein in the rod cells of vertebrate retinas. Reports of a phototaxis receptor in a eucaryotic green alga² and the three cone pigments mediating human colour vision contributed to the liveliness of the meeting.

The lateral position of the retinal group of bacteriorhodopsin has been investigated by neutron diffraction of membranes containing hydrogenated or specifically deuterated retinal (N. A. Dencher, Berlin). Peaks in neutron difference maps fit nicely with the proposed position of the C-terminal helix, which contains lysine 216, the residue joined to retinal by a protonated Schiff base linkage (R. Henderson, MRC Cambridge). X-ray and electron diffraction patterns of M412, the deprotonated species in the proton-pumping photocycle, are very similar to those of protonated BR. The extent of structural change corresponds to a movement of one or two amino acid residues by a distance of about 4 Å. A rotation or tilting of an entire helix is excluded by these data.

The configurations of key bonds in the retinal group of bacteriorhodopsin attracted much interest. Resonance Raman spectroscopy and solid-state ¹³C-NMR have provided a wealth of information concerning the precise structural changes of the chromophore during the photocycle^{3,4}. Theoretical calculations indicate that rotation about the C14–C15 single bond is severely hindered in the protonated state. This single bond is thought to be essentially free in the unprotonated state, as well as in a protonated state, with a negative counter-ion near the Schiff base nitrogen (K. Schulten, Munich). Rotation about the C14–C15 bond may be an important gating mechanism in proton pumping by bacteriorhodopsin. Fourier transform infra-red spectroscopy has shown that several carboxylates undergo

changes in the photocycle (F. Siebert, Freiburg). It seems likely that the pathway of proton pumping will be known in atomic detail before long.

Halorhodopsin, discovered several years after bacteriorhodopsin⁵, pumps chloride ions into halobacteria⁶. Three different classes of chloride binding sites have recently been identified (B. Schobert, University of California, Irvine; T. Yoshizawa, Kyoto). Because it is highly specific for halide ions, site II is probably directly involved in transport. Moreover, occupancy of this site leads to a large spectral shift of the retinal chromophore. Site I affects the *pK* of the retinal Schiff base, whereas site III is less specific and has lower affinity for chloride. The photocycle of halorhodopsin resembles that of bacteriorhodopsin apart from the absence of a deprotonated intermediate. Instead, the most blue-shifted species is thought to arise from the interaction of the transported chloride ion with the protonated Schiff base nitrogen (D. Oesterhelt, MPI Martinsried). Halorhodopsin probably transports chloride into the cell by a mechanism very similar to that employed by bacteriorhodopsin to pump proton out of the cell.

Phototaxis

Halobacterium halobium is attracted to long-wavelength visible light (the red response) and repelled by short-wavelength light (the blue response)⁷. Halobacteria reverse their swimming direction when they encounter an increase in blue light intensity or a decrease in red light intensity. The isolation of a mutant devoid of BR and HR has led to the discovery of a third light-activated retinal protein in halobacteria⁸. This pigment has a photocycle akin to that of BR and HR, but it occurs on a slower time scale, and so was initially called slow-cycling rhodopsin (SR)⁹. Later studies of SR revealed it to mediate the attractant red response (PS565). Hence, SR now stands for sensory rhodopsin.

What about the receptor for the repellent blue response? The surprise is that SR does double duty, serving as both the attractant and repellent receptor (J. L. Spudis, Albert Einstein, College of Medicine¹⁰; R. A. Bogomolni, University of California, San Francisco). Absorption of a red photon by the dark-adapted state of SR (SR587) converts it into a form (SR373) that absorbs maximally at 373 nm and leads to an attractant signal. Absorption of a blue photon by SR373 then gives rise to a repellent signal. The repellent response is much more sensitive than the attractant response. Indeed, excitation of a single molecule of SR373 appears to be sufficient

to generate a reversal-inducing repellent signal. A key experiment leading to this proposed photochromic mechanism for the dual role of SR is that the repellent response to blue light is dependent on the presence of red background illumination. An alternative view, espoused by some aficionados of halobacteria phototaxis, is that different receptor proteins trigger the attractant and repellent responses¹¹. After a spirited evening discussion, it was agreed that a mutant lacking SR is needed to settle the issue. The effects of blue and red flashes separated by different intervals should also be highly informative. My feeling is that the photochromic mechanism is basically correct, and that halobacteria have exploited the convertibility of retinal isomers to get primitive colour-sensing from a single retinal protein.

Image-forming eyes are present in four phyla — molluscs, annelids, arthropods, and vertebrates. The action of photo-excited rhodopsin (R*) is best understood in vertebrates, where it triggers a cascade that leads to the hydrolysis of cyclic GMP (L. Stryer, Stanford University; M. Chabre, University of Grenoble). In the first stage, a single molecule of R* catalyzes the exchange of GTP for GDP bound to hundreds of molecules of transducin (T), a multisubunit peripheral membrane protein¹². *Tα*-GTP then activates a cyclic GMP phosphodiesterase. Hydrolysis of bound GTP brings transducin back to the dark state. This cycle is driven by the phosphoryl potential of GTP, which is nicely partitioned between the activation of transducin and the subsequent hydrolysis of its bound GTP. The restoration of the dark state also requires that R* be deactivated, which is accomplished by the phosphorylation of multiple serines and threonines in its carboxy-terminal domain (H. Kühn, KFA, Jülich). Phosphorylated rhodopsin is then capped by a 48K protein, which probably prevents it from interacting with transducin. Infra-red light-scattering studies of magnetically oriented rod outer segments have shown that a molecule of R* activates a molecule of transducin in 1 msec, which is sufficiently rapid to enable transducin to participate in visual excitation¹³. The activation of transducin and the consequent hydrolysis of cyclic GMP are only part of the story. The challenge now is to delineate the links between this cascade and the closure of sodium channels in the plasma membrane. Calcium, is almost certainly involved, but its relationship to the cyclic GMP cascade is still an enigma.

The amino acid sequence of bovine rhodopsin and of the *γ*-subunit of transducin have been determined (Y. Ovchinnikov, Shemyakin Institute, Moscow). The sequences of bovine rhodopsin is not similar to that of bacteriorhodopsin, although there are similarities in their architecture¹⁴. Furthermore, vertebrate rhodopsin is like bacteriorhodopsin in the first few picoseconds of its photo-

*Molecular Biology of Retinal Proteins, Schloss Ringberg (near Munich), 10–14 September 1984.

chemistry. Thereafter, the role of photo-excited rhodopsin is to trigger an enzymatic cascade, whereas that of bacteriorhodopsin is to pump a proton and recycle rapidly. It will be interesting to see whether the rhodopsins of molluscs and arthropods are like those of vertebrates, as hinted at by the finding that squid rhodopsin can activate vertebrate transducin (H. Saibil, University of Oxford).

The genes for the three visual pigments mediating colour vision in humans have been isolated (J. Nathans, Stanford University) by a tour de force that began with the isolation of the gene for bovine rhodopsin¹⁵, which was then used as a hybridization probe to isolate the human rhodopsin gene¹⁶ and has now been similarly used to isolate the three genes for the colour pigments: two (probably the red and green absorbing pigments) are on the X-chromosome and the other (probably the blue absorbing pigment) is on an autosome.

Sequence analysis of the bovine and human rhodopsin genes had shown that they have the same intron-exon arrangement and that the two proteins have the same number and a 94 per cent match of amino acids. Moreover, the retinal attachment site, the two glycosylation sites, all but one of the phosphorylation sites and, most strikingly, the three loops on the cytoplasmic face of bovine rhodopsin are perfectly conserved; these loops almost certainly form the binding sites for transducin and other proteins that interact with photoexcited rhodopsin, such as rhodopsin kinase and the 48K protein. The intron-exon arrangements of the colour vision genes are like those of rhodopsin but the amino acid sequences have diverged considerably. Because the colour pigments have not yet been purified and sequenced the complete nucleotide sequences of their genes are eagerly awaited. Thomas Young, who in 1802 proposed that colour vision is mediated by three distinct receptors, would no doubt have derived enjoyment and satisfaction from knowing that their genes are now in hand. □

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Neurosciences

Spanish reflections and Cajal

from A. Claudio Cuello and Alan Cowey

IT IS no exaggeration to say that Don Santiago Ramon Y Cajal laid the foundations of modern neurobiology. Certainly he has been the most important scientist ever produced by Spain and his name still possesses some magical appeal, fifty years after his death. To mark that anniversary, the Cajal Institute of Madrid organized a meeting of neuroscientists in the elegant setting of the Consejo Superior de Investigaciones Científicas, Madrid, from September 17–20 1984.

Neuronal connectivity, which could be described as having been discovered by Cajal, was given pride of place in a session that showed with extraordinary clarity how much modern techniques of labelled axoplasmic transport and immunocytochemistry, combined with the Golgi methods used by Cajal himself, are revealing about specific connexions and their development. Regeneration in the central nervous system, another of Cajal's abiding interests, was discussed in relation to new surgical techniques, to models of how appropriate connexions are formed, and to the exciting new evidence that intracerebral grafts may not only survive, but function. A session on synaptic transmission provided clear evidence of the extent to which synaptic events are now explained in terms of the molecular structure of receptors, and the development of nerve cells can now be attributed to electrical oscillation and resonance. Even Cajal could not have foreseen the final symposium on genetic engineering in neurobiology in which several examples were presented of how it is now possible to identify genes whose expression is limited to identified neurones, and how a given gene product can influence the physiology of a cell.

It was refreshing to see so many young Spaniards eagerly following these accounts of recent developments in the neurosciences, during which there were repeated references to the outstanding contributions made by 'Don Santiago' and his followers, a measure of the permanence of his school in contemporary neurobiology. So it is ironic that Spaniards now have to look abroad to enable themselves to keep pace with developments in this field. The exodus of eminent neuroscientists from Spain started early this century and was aggravated by the Civil War. Pio del Rio Hortega left for Oxford and then moved to Argentina where he generated great interest in neurohistology. Lorento di No emigrated to the United States, where he continued his timeless work on neuronal structure. Among countless other Spanish neuroscientists distributed among their adopted countries are J. Palacios in Swit-

erland, C. Sotelo in France, M. Marin-Padilla, A. DeBlas and M. Nieto Sampedro in the United States, and F. Cervero in Britain.

Will any of these distinguished scientists ever return to the homeland and contribute to the development of neurosciences in the land of Cajal? Probably not. The most realistic possibility for Spain to regain a proper international place in neurobiology would be for young neurobiologists to be offered good possibilities to carry out advanced research projects at home. A good number of Spanish scientists are currently trained abroad and some of Spain's more mature scientists are making tremendous efforts to expose themselves to new ideas and techniques. For all this to prosper there is a need for a firm intention to consolidate these gains, together with a sustained effort to finance the fresh demands these investigators will make. If the sometimes passionate words of the President of the Consejo Superior de Investigaciones Científicas and the Ministers of Education and Health expressed on the occasion of Cajal's anniversary are taken literally, then we can expect a revival of the Cajal legend to have a positive impact on Spanish neuroscience. But a sincere intention will need to be behind the rhetoric. Cajal's gigantic figure should serve Spain as a source of continuous inspiration and not just simply a glorification of the past. □

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A METEOR VISIBLE IN THE DAYTIME

At Waterpark, just below Waterford City, about 4 p.m. on October 15, my attention was attracted by a flash of increased light. Looking up, I saw in the south-south-west, about half way between the horizon and the zenith, a bright meteor slowly sailing nearly due west, its apparent size about half of the full moon, intensely white in colour at the centre, passing into blue at the circumference. It described a low arc, and was in sight for several seconds, leaving a trail of indigo blue with lighter luminous edges. The meteor disappeared behind some clouds which concealed the sun at a considerable altitude above the horizon.

From *Nature* 31, 102, 4 December, 1884.

Seismology

First results from Japanese network for earthquake prediction

From Y. Okado.

A VERY extensive high-quality digital network for micro-earthquake and ground-tilt observation has recently been completed in the south Kanto-Tokai area surrounding Tokyo. The first scientific results are now appearing and allow, among other things, a remarkable three-dimensional picture of the earthquake hypocentre distribution to be produced. The distribution maps out the boundaries of the three plates that meet under this region of Japan^{1,2}.

The new network — perhaps the most extensive in the world — has been built primarily to aid the prediction of earthquakes in an area that contains a large fraction of Japan's industry and population. Construction began in 1978 aided by the passage of the Large Scale Earthquake Countermeasures Act which provides for the preparation of specific measures to be taken when an earthquake of magnitude 8 or more is predicted to be imminent. The network will also provide many data of great scientific interest because it is monitoring seismic activity in the unique triple junction of the Eurasian, Philippine and Pacific Plates. It is at the boundaries between these plates that great earthquakes have repeatedly occurred in the past. It is thought that an interplate

earthquake with a magnitude of 8 or more will occur in the Tokai region in the near future and a long-term prediction has already been issued to the general public.

The network, which has the National Research Centre for Disaster Prevention (NRCDP) in Tsukuba Science City at its hub, consists of 71 stations (Fig.1) including three deep borehole observatories, 48 borehole stations approximately 100 m deep and 20 surface stations covering an area of approximately 350 × 150 km² with an average distance of 25 km between adjacent stations. Among them, three deep borehole observatories which reach the basement rock at depths of 3,510 m, 2,330 m and 2,710 m, were completed in 1980 in and around Tokyo to overcome the artificial noises at the surface in the capital area. Most of the stations are equipped with a three-component set of velocity-type seismographs with a natural frequency of 1 Hz. A two-component borehole tiltmeter is sited at 23 stations, some of which are also equipped with special instruments such as three-component borehole strainmeters, acoustic emission sensors, ground-water-level meters and geochemical sensors.

All the observed data are digitized and transmitted with the pulse code modu-

lation method through telephone lines to NRCDP. The telemetry system uses various modern techniques such as automatic network timing (ANT) and wide dynamic range quantization (78 dB for 80 Hz sampling seismic data and 96 dB for 1 Hz sampling tilt data), and provides a density of transmission — 9,600 bits per second — that has never before been achieved by a conventional telemetry system. ANT uses a set of subclocks which generate echo-back signals to monitor continuously the turnaround times of each station and automatically adjust their midpoints to coincide with a master clock. By this method, the data sampling at all stations can be synchronized with an error of less than a few milliseconds and the problem of telemetry delay-time can be solved.

At the centre in Tsukuba, the outputs of all vertical seismograph and tilt data are continuously monitored on long-term chart recorders, while other components of the seismograph are recorded on different charts by the trigger method. Stress is put on quick and reliable data processing in order to allow short-term prediction. One computer system, directly connected with the telemetry system, carries out the filing

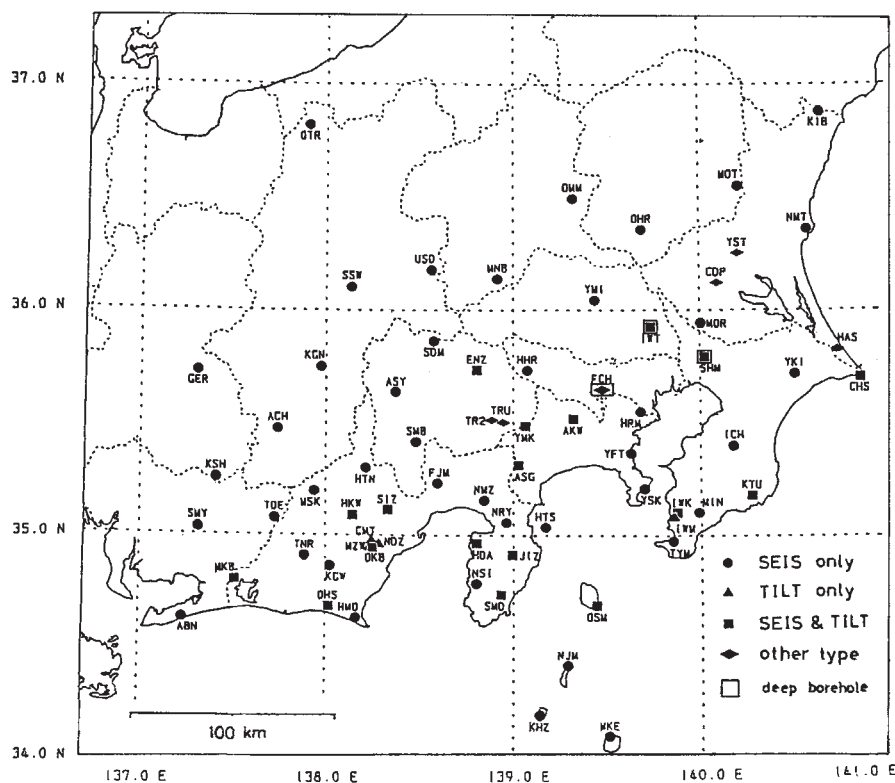


Fig.1 National Research Centre for Disaster Prevention network for microearthquake and ground tilt observation (the southernmost station HCY at latitude 33.1°N is omitted).

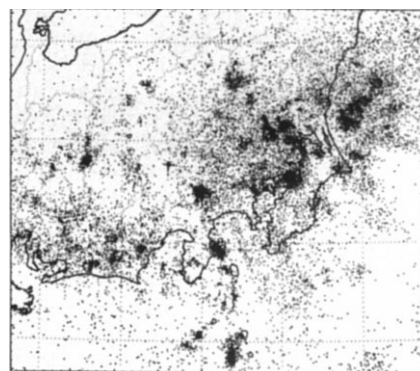


Fig.2 Distribution of earthquakes in the Kanto-Tokai region located by NRCDP in the 5 years from 1 July 1979 to 30 June 1984. The plot shows about 28,000 earthquakes, most less than 3 in magnitude. (Latitude and longitude as on Fig.1).

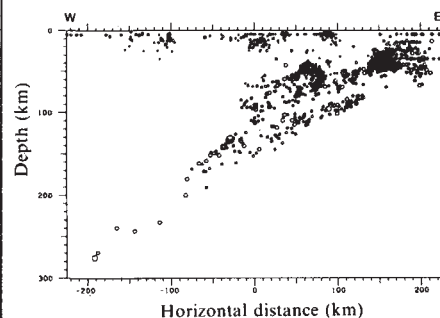


Fig.3 East-west cross section of hypocentres of 1,515 earthquakes detected in the period 1 November 1982 to 31 October 1983 within 25 km on either side of the 36.25°N line. The Pacific plate is plunging underneath the Eurasian plate, while the Philippine Sea plate is being subducted from the south and collides with the Pacific plate at a depth of about 60 km making an 'earthquake nest' under the Kanto region.

of observed data and monitors the crustal activity on a real-time basis. Further data-processing steps, including hypocentre determination by rationalized semi-automatic methods, are executed by a second computer system.

Routine work for hypocentre determination began as soon as the first parts of the system were completed in July 1979. Every day around twenty earthquakes are located, most of them micro-earthquakes with magnitude around two. Figure 2 shows the hypocentres in the Kanto-Tokai area which have been detected by our network in the past 5 years and figure 3 shows the hypocentres in a vertical section through 36. 25° N (ref.3). The vertical section clearly shows the Pacific Plate diving down underneath the Eurasian Plate upon which the main Japanese island

stands. The precise study of three-dimensional hypocentral distribution and focal mechanisms based on a large amount of accurate data leads us to an excellent image of the plate configuration in this complex region⁴. Also we have been able to measure a precursory tilt change in the 18 days preceding an earthquake that was of magnitude six and thirty one km away from the station⁵. □

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Earthquake prediction

Evaluating hydrological and geochemical anomalies

from Chi-Yu King

THE 27th International Geological Congress was held in Moscow from 4–14 August 1984 and, like many other recent geoscientific meetings, included earthquake prediction as a theme. Among the papers presented were five based on hydrologic and geochemical studies by Soviet scientists, providing some current views of the Soviet effort in this relatively new field of study. While some scientists consider the hydrochemical and geochemical methods highly effective for short-term prediction, others remain sceptical.

Substantial efforts to predict the time, location and magnitude of earthquakes with reasonable precision began about two decades ago in the Soviet Union, Japan, China and the United States. The Soviet studies were triggered by the destructive Khait earthquake of 1949 in the Garm region of Tajikistan. Hydrochemical and geochemical methods began to draw attention when the 1966 Tashkent earthquake was found to have been preceded by anomalous radon changes in groundwater. Currently, a total of 240 wells are being monitored at 83 stations in practically all USSR seismic regions. About 40 parameters are being measured, including water level (or flow rate), temperature, electric conductivity, ion content, isotopic ratios (such as D/H, ¹⁸O/¹⁶O), and the presence of gases (such as Rn, He, H and Hg vapour) either dissolved in groundwater or emanating from the ground. Many anomalies have been recorded before large earthquakes as far away as several hundred kilometres. The amplitude, duration and spatial extent of the anomalies are generally found to increase with earthquake magnitude and hence provide a means of prediction. Some earthquakes are even

claimed to have been predicted on the basis of hydrochemical geochemical and other data, but insufficient data were shown to permit a critical assessment of such claims.

Are the hydrochemical and geochemical changes produced by earthquake-related fluid movements in the earth — such as increased upward flow of deep-seated water and gas, flow of water from one aquifer to another through tectonically developed cracks in the barrier between them or movement of pore fluid squeezed out of surrounding rocks into well waters? It is conceivable. But these changes may also be the result of other environmental factors, such as rainfall, barometric pressure and groundwater pumping. Consequently, it is difficult to distinguish true earthquake-related anomalies from noise. Identification of anomalies is complicated further by the local geology of the monitoring sites, the lack of detailed understanding of fluid movement in the ground and data contamination by errors introduced in sampling and measurement. Most researchers in the Soviet Union and elsewhere agree that, to pursue earthquake prediction properly, it is necessary to use data recorded over a number of years with a variety of different instruments.

China initiated a massive earthquake-prediction program shortly after the 1966 Xingtai earthquake, which was the first of nine large earthquakes (magnitude ≥ 7.0) that devastated many areas on the mainland between 1966–76. The Chinese effort has mobilized tens of thousands of people, both professional and amateur, and has used many different methods, both modern and traditional. About 4,000 wells have been used for hydrochemical and geochemical monitoring, but only a few per

cent of them have been found useful. Because of the timeliness of the effort, a large number of scientific data and eyewitness accounts have been collected, many of which describe hydrochemical and geochemical changes. As in the Soviet Union, numerous pre-earthquake anomalies have been recorded at epicentral distances of up to several hundred kilometres in China and some of the data in combination with macroscopic phenomena and other kinds of data have been used successfully to predict several earthquakes including those in Haicheng in 1975 and Songpan in 1976. However, because of the aforementioned difficulties, it will not be possible to assess critically the relation of many of the reported anomalies to subsequent earthquakes until more data are published.

The Japanese earthquake-prediction program was implemented shortly after the destructive 1964 Niigata earthquake. Hydrochemical and geochemical observations began in 1973 and are carried out mostly in the Tokai region by the Geological Survey of Japan and several universities, with the help of some amateur groups. Anomalous changes have been reported in association with some earthquakes including that in Izu-Oshima-Kinkai in 1978.

In the United States, research on earthquake prediction has intensified since the Alaskan earthquake of 1964. A national program was implemented in 1977, with field effort concentrated in geophysical (especially, seismic and geodetic-strain) measurements along the San Andreas fault system in California. US hydrochemical and geochemical studies have been relatively few and have mostly involved simple inexpensive techniques and about 70 existing wells and springs, which may not always be suitable for earthquake prediction. There have been some moderate earthquakes in California in the past years, but few reliable hydrochemical, geochemical or geophysical anomalies were recorded. Some attribute this to the tectonic environment: the San Andreas fault system is characterized by simple shear movement with little stress concentration, whereas seismic regions in the Soviet Union and China are characterized by complex continental structure in a highly compressed state and earthquake-related anomalies may occur at the structurally singular points where stresses concentrate. Most of the earlier US results were published in the *Journal of Geophysical Research* in June 1980 and *Geophysical Research Letters* in May 1981.

Approximately 30 papers on earthquake hydrology and chemistry, including some presented at the 27th International Geological Congress, will be published in early 1985 in a special issue of *Pure and Applied Geophysics*. □

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Immunology

Exclusive immunoglobulin genes

from Frederick W. Alt

ON PAGE 517 of this issue, Ritchie, Brinster and Storb describe an elegant experiment¹ that provides strong support for one mechanism of the phenomenon known as allelic exclusion, which has traditionally been as puzzling an aspect of immunoglobulin genes as has their ability to specify a limitless diversity of antibodies.

Allelic exclusion, which is unique to immunoglobulin and related genes, means simply the expression of only one of the pair of genes that have the potential to specify a given chain of the immunoglobulin molecule^{2,3}. Although the function of allelic exclusion is still debated⁴, the simplest view is that the generation of mono-specific B-lymphocytes is an important pre-requisite to an immune system whose specificity is based on clonal selection, and that a mechanism has evolved to ensure such specificity.

From the molecular analysis of the immunoglobulin genes, it at first seemed that the explanation of both the generation of antibody diversity and allelic exclusion might lie in the fact that the variable (*V*) region of the antibody molecule is encoded in separate segments of DNA that are brought together in different combinations by rearrangement in the course of B-cell differentiation. This process accounts for much of the diversity of antibodies and, being error-prone, can be shown to result in the assembly of a non-functional gene. Thus it was conceivable that the probability of a functional rearrangement is low enough to make it very unlikely that two functional alleles would be generated. This explanation of the phenomenon of allelic exclusion is known as the stochastic model. There were, however, always problems with the simplest form of this model; Ritchie *et al.* now provide definitive evidence for an alternative model, known as the regulated model. By transfecting early mouse embryos with an already rearranged gene for the kappa (κ) light chain — one of the immunoglobulin chains — they have produced transgenic mice that express these genes in their B lymphocytes. To understand the implications of these new data, some background is necessary.

Each B lymphocyte displays on its surface an immunoglobulin molecule of a single specificity which is composed of a complex of two identical heavy chains and two identical light chains, the amino termini of which interact to generate the specific antigen binding pocket. The variable region of each light chain is encoded by a variable (*V*) gene segment and a joining (*J*) segment which are separate in the germ line DNA but are assembled somatically to form the complete variable region gene. There are two families of light-

chain genes, kappa and lambda, only one of which is expressed in any one cell. Immunoglobulin heavy-chain variable genes are also assembled from separate segments but in this case, there are three: the *V*, *D* (diversity), and *J* segments; the *D* to *J* complex is formed first and a *V* segment is added later to form the complete gene. (for review of *V* gene assembly see ref. 5). During B-cell differentiation, heavy chain gene assembly and expression generally precedes that of light-chain genes⁶.

Early speculation on the mechanism of allelic exclusion favoured a model in which only the 'allele' would be rearranged in a given B-cell clone, the other 'allele' remaining in germline configuration. But it quickly became apparent that within a population of normal immunoglobulin producing B-lymphocytes, significantly more than half of the κ light-chain alleles and nearly all of the endogenous heavy-chain alleles were rearranged at the *J* locus^{5,8}. Further analysis, however, revealed that one of the rearrangements was usually non-functional; for example, because of flexibility in the exact point of joining, the different segments sometimes end up in different translational reading frames. This led to so-called stochastic models for allelic exclusion, which postulated that because of the preponderance of non-functional rearrangements over function rearrangements, the probability of achieving two functional rearrangements in one cell was very low.

Regulated model

Such models, however, do not easily explain either why a significant percentage of the 'second' alleles in κ -producing cells are in the germ line configuration or why allelic exclusion is almost always observed. Moreover, in κ light chain-producing cells with dual *VJ* rearrangements, both rearranged genes may be transcribed, and in several characterized cases both transcripts have been shown to be translated. Yet only one transcript in each case encodes a light chain that combines with the cellular heavy chain to produce the immunoglobulin molecule⁷⁻⁹. This led to an alternative hypothesis; namely that allelic exclusion is effected by a 'signal' that is produced when rearrangement generates a light chain capable of combining with the pre-existing cellular heavy chain to form a functional immunoglobulin molecule. Such a signal would feed back and prevent further rearrangement. According to this model, if the first rearrangement were 'functional', a second rearrangement would be prevented — leading to a cell with one unrearranged κ allele. Were the first rearrangement to be 'nonfunctional', then a second could occur, — yielding a cell in which both alleles

are rearranged. Although this 'regulated' model seems to account for the various patterns of immunoglobulin gene rearrangement or expression that have been observed in tumour cells or normal cell populations, until now direct evidence has been lacking.

The transgenic mouse system provides an ideal experimental test of the predictions of such a model. In order to analyze the κ chains and the rearrangement of their genes, Storb and her colleagues made hybridomas from individual B lymphocytes obtained from the spleens of the transgenic mice. The regulated model predicts that expression of a transgenic κ chain in association with heavy chains in the B-cells (represented by individual hybridomas) of the transgenic mice, would prevent rearrangement of the endogenous κ genes. This is precisely consistent with the data from the hybridomas. Likewise, the regulated model predicts that if cells secrete an endogenous κ chain in the form of complete immunoglobulin, then the introduced gene will either not be expressed or will not associate with the endogenous heavy chain; several examples of the former phenomenon were observed. Most significantly, Ritchie *et al.* have not found any hybridoma that secretes the inserted κ -gene product in complete immunoglobulin and which has rearranged its endogenous κ genes. This is totally consistent with a regulated model of light-chain allelic exclusion.

A variety of evidence suggests that heavy-chain allelic exclusion is also mediated by a cessation of *V* assembly once a functional *VDJ* gene is assembled and expressed; among the evidence is the large percentage of frozen *DJ* intermediates at the 'non-functional' heavy-chain allele in populations of normal immunoglobulin-producing B-cells¹¹. Complete and active heavy chains have also been introduced into the germline of transgenic mice and shown to be expressed only in the appropriate lymphoid tissues¹²; in experiments designed to investigate the effect of the expression of the introduced gene on endogenous heavy chain gene rearrangement, pre-B lymphoid cell lines established from the bone marrow of the mice by transforming the cells with the Abelson murine leukaemia virus. Such cell lines, when generated from normal mice, almost always have dual rearrangements of both *J* alleles¹⁰. However, D. Weaver and D. Baltimore (personal communication) find that in cells from mice which express the heavy chain of the introduced gene, a substantial proportion of the heavy-chain alleles remains in the germline configuration. Although this striking finding is most easily interpreted as supporting a regulated model of heavy-chain allelic exclusion, other explanations — such as a difference in the populations of potential viral targets in the transgenic and normal mice — have not been excluded.

Thus the use of mice harbouring introduced genes has provided strong evidence for the concept that heavy-chain

and light-chain allelic exclusion is very strictly regulated and not simply a by-product of error-prone rearrangement. Although the existing evidence implicates the complete heavy chain and the complete immunoglobulin molecule, respectively, as the primary signals for heavy-chain and light-chain allelic exclusion, we are still far from understanding the molecular mechanism by which such signals are transmitted. Further insight may come from the observation that there are no consistent differences between the recombination/recognition sequences that flank the various heavy-chain and light-chain gene segments that are assembled at different points in B-cell ontogeny⁵. These findings suggest that all recombination events may be mediated by the same 'recombinase system'. If so, both allelic ex-

clusion and ordered rearrangement may be mediated by the accessibility of the substrate gene segments to a common recombinase rather than modulation of the activity of segment-specific recombinases. □

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Tumour development

Oncogenes in transgenic mice

from Douglas Hanahan

THE study of oncogenes is increasingly directed toward identifying the functions of their products and the molecular basis for the transformations they effect. One approach is to insert cloned (and sometimes deliberately altered) oncogenes into the germ line of an organism to establish their effect in that context. Two groups have recently illustrated how this approach can provide information on oncogene function and the development of cancers, whilst a third has demonstrated that a proto-oncogene can induce embryonal cells to differentiate.

In the October issue of *Cell*, Stewart, Pattengale and Leder¹ describe the occurrence of mammary tumours in transgenic mice which developed from one-cell embryos injected with hybrid oncogenes. The fusion genes comprised the long terminal repeat (LTR) sequence of mouse mammary tumour virus (MMTV) linked to various portions of the mouse *c-myc* proto-oncogene. The LTR carries transcriptional control sequences, including a transcriptional enhancer element and sequences which mediate induction of transcription by glucocorticoid hormones^{2,3}. The *c-myc* gene is the cellular homologue of the oncogene carried by MC29, an avian retrovirus², and is itself activated to participate in oncogenesis by retroviral insertion⁴ and by chromosomal translocation into the immunoglobulin locus^{5,6}. The *c-myc* gene product is a nuclear protein which binds DNA *in vitro*; its expression is apparently cell-cycle regulated, being strongly induced during the transition from the G₀ to the G₁ stage⁷.

Twelve of the thirteen transgenic mice obtained by Stewart *et al.* express the fusion gene in their salivary glands, several produce fusion gene transcripts in a few other tissues, and one shows constitutive

expression in all tissues. Each of the three females examined expressed the transgene in mammary tissue. The product of the fusion gene is the authentic mouse myc protein, which means it is difficult to distinguish from the product of the endogenous *c-myc* gene, but which allows the effects of quantitative differences in the normal protein to be examined.

Two of the original transgenic females developed spontaneous mammary adenocarcinomas while they were lactating. The tumours arose in one of the ten mammary glands of each mouse. There was no obvious DNA amplification or rearrangement in the tumour tissue. Fusion gene transcripts were abundant in the tumour relative to other tissues, as well as to transcripts from the endogenous *c-myc* gene. The development of mammary tumours was heritable from one of the two females, whose three transgenic female progeny developed similar mammary tumours during pregnancy. It is notable that the fusion gene produced tumours only in the tissue that is the primary target for MMTV. However, its penetrance is obviously incomplete, since the development of the cancer occurs only during pregnancy, does not arise in every mammary gland, and occurs in only a fraction of the independent strains harbouring the fusion gene. This implies that hormonal balance is in some way important and furthermore, that secondary events are necessary to elicit transformation. There were no discernable consequences of transgene expression on salivary glands of any of the twelve founder mice, nor on any other tissues found to be expressing the fusion gene.

In a somewhat analogous set of experiments, Brinster *et al.*⁸ have previously reported the transfer to the germ line of mice of the simian virus (SV) 40 T-antigen

'oncogene' (in the form of the early region of the viral genome in plasmids that also included either thymidine kinase or growth hormone genes^{9,10}, in both cases fused with, and so under the control of, the promoter of the mouse metallothionein gene). Transgenic mice were produced at frequencies comparable with those obtained with the fusion genes alone, which indicates that the presence of the SV40 T-antigen 'oncogene' is not necessarily deleterious to prenatal development. About 25 per cent of the transgenic mice develop tumours of the choroid plexus, a layer of epithelial cells which lines the brain and forms the blood-brain barrier. And virtually all of the original transgenic mice died prematurely. This phenotype is heritable, and is transmitted along with the T-antigen gene, which includes the normal SV40 transcriptional control regions.

The expression of the acquired transgene has several notable characteristics. The choroid plexus tumours, and cell lines derived from them, contain significant levels of SV40 T antigen and its corresponding message. However, no expression was detected in total brain tissue prior to tumorigenesis, although the presence of small quantities in the choroid plexus cannot be excluded. Furthermore, the metallothionein fusion genes are rarely expressed in most of these mice, which is in marked contrast to their frequent expression in transgenic mice that lack the accompanying SV40 early region^{9,10}.

Taken together these results suggest that the injected DNA (T-antigen gene plus metallothionein fusion gene) is inactivated early in development in some stable but reversible manner. There is a precedent for the stable inactivation and subsequent reactivation of the SV40 early region in cultured cells: a cell line carrying 25–50 copies of the intact SV40 early region, which produced no detectable T antigen following initial cloning and continued passage, subsequently expressed T antigen in an occasional cell, and when such cells were subcloned they were found to produce T antigen in a clonally stable manner¹¹. Several of the choroid plexus tumours and cell lines derived from them seem to contain amplified SV40 sequences, which suggests that amplification (and rearrangement) may be one mechanism for SV40 gene activation. The concepts of stable germ-line inactivation and subsequent somatic reactivation have been well documented in mouse strains harbouring genetically transmitted proviruses of murine leukaemia virus^{12,13}. In the case of transgenic mice, the inactivation affects not only the SV40 early region but also the normally active metallothionein fusion gene to which it is linked. The fact that only the choroid plexus is transformed may be accounted for by the tissue specificity of the SV40 transcriptional enhancer element.

In the studies of both Stewart *et al.* and Brinster *et al.*, the presence of an oncogene

in the germ line is not sufficient to produce a simple dominant phenotype. Rather, additional rare changes seem to be required for the oncogene to produce tumours. This has similarities to the evolving view of naturally-occurring cancers^{11,15}, where multiple events are most probably necessary to create the dominance that converts a normal cell into a tumour cell, and to explain the slow onset of transformation.

The production of transgenic mice harbouring new oncogenes requires that such genes do not perturb prenatal development. The work of Müller and Wagner¹⁶ nicely suggests that not all oncogenes do affect differentiation. They have transferred the *fos* proto-oncogene into F9 embryonal carcinoma cells, where it induces characteristic terminal differentiation.

F9 cells were originally established from a teratocarcinoma and have many qualities in common with cells of the early mouse embryo, as demonstrated by their ability to form aggregation chimaeras with normal embryo cells and their representation in somatic tissues of mice that develop from the chimaera. F9 cells can be induced by the combination of retinoic acid and dibutyryl cyclic AMP to undergo a series of alterations, resulting in epithelial-like cells that cease to divide and show many characteristics of authentic endoderm tissue¹⁷.

The *c-fos* gene, the cellular homologue of an oncogene (*v-fos*) found in several acute transforming viruses, produces a nuclear phosphoprotein¹⁸ that is expressed at very high levels in extra-embryonic tissues, especially amnion and visceral yolk sac¹⁹. When F9 cells are cotransfected with *c-fos* and an unlinked neomycin gene, which confers resistance to the drug G418, two classes of drug-resistant colonies arise. One consists of proliferating cells with normal F9 morphology. The other comprises enlarged, flat cells that assume an epithelial morphology and cease to proliferate. The flat cell colonies contain high levels of *c-fos* DNA, mRNA and protein.

The morphologically-altered cells develop an ordered array of intermediate filaments that carry several antigens characteristic of endoderm tissue. However, these cells do not produce either laminin or

α -fetoprotein, which are found associated with parietal and visceral endoderm, respectively. Fibronectin and type IV collagen are both detected in these cells — collagen is normally present in endoderm, while fibronectin is not. In contrast, retinoic acid induces the production of the intermediate filament network and type IV collagen, as well as either laminin or α -fetoprotein, but not fibronectin. Thus, the introduction and expression of the *c-fos* gene results in specific changes that are distinguishable

from those resulting from chemical induction. The ability to induce events characteristic of differentiation through the expression of a single transferred gene provides new opportunities for studying the role of gene expression (and its regulation) in some of the specific changes that are involved in complex this differentiation pathway. □

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Surface chemistry

Removing the black magic

from J.B. Pendry

MUCH of our chemical industry would not be economic without the presence of a catalyst, acting to short circuit energy barriers to the desired transmutation of one species of organic molecule into another and to discriminate against unwanted products. Frequently, the organic reactants are adsorbed on the surface of finely divided particles of a catalytic transition metal, the complex electronic structure of which offers a variety of possibilities for bonding, thus facilitating rearrangement of the reacting species. The key to understanding the catalyst's function lies in the configuration of the reactant molecules at the surface — that is in their crystallography. So thin and delicate is the monolayer of reactants that it is far from trivial to determine their crystallographic structure. It has only been in the last decade that surface crystallography can be said to have existed. At the International Conference on the Structure of Surfaces, 13-16 August, in Berkeley, California, Roland Koestner, Michel Van Hove and Gabor Somorjai of the university there, reported a major advance in crystallographic sophistication.

Apart from the detailed location of the hydrogen atoms, the Berkeley group was able to solve the complete crystallographic structure of hydrocarbon monolayers on platinum and rhodium surfaces formed by the adsorption of C_nH_{2n} , where n varies from 2 to 4. A great variety of structures form under different conditions. For example, it is possible to persuade one of the terminal carbon atoms to discard its hydrogen atoms in favour of bonding to the metal surface, settling into a three-fold hollow site available on the (111) single crystal surface used in the experiment. Some of the hydrogens are claimed by the unsaturated bonds further down the chain, forming the alkylidyne species $M_3C-(CH_2)_{n-1}-H$, which stands as close to vertical to the surface as the crooked C-C-C bond angle will allow (M_3 represents the three surface atoms to which the terminal carbon bonds).

For propylidyne and butylidyne species, a further wrinkle to the structure was

untangled. The crooked bond angles mean that $C-CH_2-CH_3$ adopts a configuration in which the $C-CH_2$ bond is perpendicular to the surface, whilst the CH_2-CH_3 bond points in some direction away from the surface. At low coverages and high temperatures this arm swings around in a random manner, but at higher coverages and lower temperatures free rotation is prevented by interaction with neighbours and an ordered structure results with the arms neatly folded into an ordered structure.

How was the Berkeley group able to make such precise statements about the surface crystallography? As with most surface experiments, several techniques were employed to cross check conclusions, but the most important was low energy electron diffraction. Electrons with energies around 150 eV have a wavelength of around 1 Å, like X rays. Unlike X rays, they interact strongly with matter giving good surface sensitivity. To obtain structural information from their diffraction patterns, a sophisticated theoretical interpretation is necessitated but the Berkeley group has achieved a level of sophistication that had been thought impossible. For butylidyne on platinum, with a $(2\sqrt{3} \times 2\sqrt{3}) R30^\circ$ unit surface cell, the coordinates of about 50 atoms had to be correctly included to an accuracy of about 0.1 Å.

Just as the journey from the original application of X rays to the structure of DNA was a long and difficult one, so there is a great contrast between the early determinations of coordinates for single atoms at surfaces, and the present work, although even butylidyne on platinum falls short of the complexity of DNA. New possibilities for studying complex systems are opened. In particular we are brought closer to an atomistic understanding of how reactions proceed at surfaces, which help to make the evolution of new higher yielding catalysts more a matter of the science of design than the black magic of trial and error. □

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Origin of the Solar System dust bands discovered by IRAS

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Measurements of longitudinal variations in the brightness and the latitude of the Solar System dust bands observed by the Infrared Astronomical Satellite determine the orbital elements of the particles involved. They may also allow discrimination between cometary and asteroidal models of the origin of these bands and even perhaps the zodiacal dust complex.

ZODIACAL light is observed as a faint glow in the west after sunset and in the east before sunrise. Conventionally, the debris of short-period comets is thought to give rise to the zodiacal cloud, the grains of which are responsible for zodiacal light. The sizes of particles emitted by comets^{1,2} are similar to those thought to scatter the zodiacal light^{3,4}, while the association of meteor streams with known comets^{5,6} demonstrates that comets inject some grains of diameter $\geq 1 \mu\text{m}$ into the interplanetary complex. In addition, the orbital elements of short-period comets generally^{3,7-9}, but not completely¹⁰, match what is known about the trajectories of interplanetary dust. A large fraction of interplanetary dust grains have low densities and airy structures similar to those expected for volatile-rich cometary particles which form in a low-gravity environment^{7,11,12}.

This hypothesis is, however, inconsistent with data showing that short-period comets are an inadequate source of debris^{10,13,14}, delivering only a few per cent of the mass needed to replenish that lost from the zodiacal complex through self-collisions and by orbital collapse due to Poynting-Robertson drag. To avoid this conflict, it has been proposed that asteroids^{10,15} rather than comets primarily supply the cloud. Alternatively, the zodiacal cloud may be episodic, being supplied intermittently by the capture of an abnormally large comet into a short-period orbit^{10,13}. A further explanation is that the current cometary mass input has been seriously underestimated because comets eject most mass in the size range of pebbles or boulders, which are difficult to detect. Infrared Astronomical Satellite (IRAS)^{16,17} and radar observations¹⁸ of macroscopic cometary debris support this possibility. IRAS observations of the zodiacal cloud may help decide between the above explanations. In particular, we demonstrate here that the Solar System dust bands, which IRAS showed to be superposed on top of the much stronger zodiacal signal, may be due to asteroid families; asteroidal dust could thus be a significant contributor to the remainder of the zodiacal signal. Unfortunately, the IRAS dust bands can also be interpreted as indicative of episodic cometary injection, where the latitudes of the bands would correspond to the inclination of the comet at the time of injection.

We show here that distinctive longitudinal variations in thermal flux and mean latitude can be used to determine the typical orbits of the grains comprising the Solar System bands. In particular, we predict how the bands should vary if they are debris associated with the three principal asteroid families. Based on these ideas, IRAS observations (after substantially more data analysis) may allow discrimination between asteroidal and cometary origins of the dust bands and, perhaps, of the entire zodiacal cloud.

Dust bands and asteroid families

IRAS discovered three comparatively narrow bands of far-infrared emission superposed on the spatially broad zodiacal background^{19,20}. The most prominent band lies in or near the ecliptic and is flanked by two other bands $\sim 10^\circ$ above and below it (see Fig. 1). These bands of dust have colour temperatures of 165–200 K. The derived distances of 2–3 AU suggest that they are approximately at the distance of the main asteroid belt and may be the results of collisions between asteroids¹⁹.

Hirayama's asteroid families²¹, defined on the basis of clustering in orbital parameter space (semimajor axis a , proper eccentricity e' and proper inclination I'), are also considered to be collision products. We show first that the latitude distributions of the asteroids in the three most prominent Hirayama families (Koronis, Eos and Themis) match the structure of the IRAS Solar System dust bands. The distribution of the main belt asteroids in the $a - \sin I'$ plane is shown in Fig. 2. The Koronis, Eos and Themis families are prominent in this plane and are equally well-clustered in the $a - e'$ plane (see Table 1). There are no other families, except perhaps the multiple Flora families at 2.2 AU, that are both as well-populated and as tightly bunched in orbital parameter space as these. The ages of the families are unknown, but because the longitudes of the proper nodes, Ω' , are randomly distributed and there are no correlations between Ω' and the nodal rates, $\dot{\Omega}'$, their ages are certainly $> T = 2\pi/\delta\dot{\Omega}' \approx 10^6$ yr, where $\delta\dot{\Omega}'$ is the observed dispersion in $\dot{\Omega}'$ (see Table 1). If the families are the products of chance collisions and if asteroidal orbits are stable over the lifetime of the Solar System, then the presence of only three or four prominent families indicates that their ages are $\sim 10^9$ yr. We can make no definitive statements about the long-term stability of Solar System orbits: planetary perturbations may disperse the orbital elements of family members in a time short compared with the age of the Solar System, in which case the families could be quite young. This would also be the case if comminution of intermediate size asteroids were important.

The distribution in latitude (integrated over all longitudes) of particles associated with the prominent families shown in Fig. 3, was obtained by distributing 50 test particles uniformly in time (or mean anomaly) on the orbits of each of the N members of each family. The central double spike in the composite distribution is due to the low-inclination families, Koronis and Themis, and has a total width (FWHM) of $\sim 4^\circ$. The wings of the distribution at $\pm 10^\circ$ are due to the Eos family, and arise because asteroids in inclined orbits spend a disproportionate amount of time at the extremes of their vertical

Table 1 Proper elements of Hirayama families

	N	a (AU)	e'	I' (deg)	$\dot{\Omega}'$ (arc s yr^{-1})	T (yr)
Koronis	42	2.875 ± 0.018	0.049 ± 0.006	2.12 ± 0.08	-66.7 ± 1.4	9×10^5
Eos	74	3.015 ± 0.006	0.071 ± 0.008	10.20 ± 0.28	-73.2 ± 0.6	2×10^6
Themis	62	3.136 ± 0.025	0.152 ± 0.009	1.42 ± 0.22	-104.4 ± 3.7	4×10^5

harmonic oscillations. Hence, a set of orbits with a common inclination and randomly distributed nodes will give rise to two apparent bands of particles symmetrically disposed above and below the mean plane of the system²⁰. There is some evidence that the central spike in Fig. 1 is a double spike; confirmation of this would demonstrate that this feature is associated with particle orbits with non-zero mean inclination and would rule out the alternative possibility that it is associated with dust which has settled to the invariable plane of the Solar System.

Size-frequency distributions

If the IRAS dust bands are located at ~ 3 AU, then from their optical depth $\sim 10^{-8}$ (ref. 19), we calculate that their total apparent area is $\sim 2 \times 10^{19} \text{ cm}^2$. The present population of small asteroids is probably the product of catastrophic collisions of larger asteroids²²⁻²⁴. The size-frequency distribution of these collision products can be described by a cumulative power law of the form

$$N(r) = \frac{1}{3(q-1)} \left(\frac{r_0}{r} \right)^{3(q-1)} \quad (1)$$

where $N(r)$ is the number of asteroids with radii $> r$ and r_0 is a constant. The theoretical equilibrium solution^{25,26} has a population index $q = 1.837$, a value which is supported by the observed distributions of small asteroids in both the McDonald and the Palomar-Leiden surveys²⁵⁻²⁷.

Since $5/3 < q < 2$, the total area, A , is dominated by contributions from the smallest particles of radii $\sim r_{\min}$, where $r_{\min}$ is the lower cutoff of the size distribution, whereas the total volume, V , is dominated by contributions from the larger asteroids. If we write $V = (4/3)\pi R_e^3$, then

$$A = \frac{\pi r_{\min}^2}{(3q-5)} \left(\frac{r_0}{r_{\min}} \right)^{3(q-1)} \quad (2)$$

and

$$r_0 = R_e [3(2-q)]^{1/3} \left(\frac{r_0}{r_{\max}} \right)^{2-q} \quad (3)$$

where $r_{\max}$ is the radius of the largest asteroid in the population.

The albedos of $\sim 40\%$ of the asteroids in the prominent Hirayama families are known²⁸, and we have used them to determine the mean albedo peculiar to each family. By assigning this mean value to those asteroids without measured albedos, we have calculated the total volume of each family (and hence R_e) from the absolute magnitudes²⁹ of the asteroids in that family. The values of r_0 calculated from the observed volumes are shown in Table 2, and the resulting power-law distributions [equation (1)] are compared with the observed cumulative distributions in Fig. 4.

The size distribution of asteroidal particles cannot be extrapolated to arbitrarily small sizes, as very small particles are destroyed by fast-moving particles associated with the cometary flux¹⁵. Dohnanyi¹⁵ estimates that asteroidal particles dominate the population of small particles only for $r > 1 \text{ mm}$. If $r_{\min} = 1 \text{ mm}$, then we calculate that the total area A of the prominent Hirayama families is $\sim 10^{19} \text{ cm}^2$ (Table 2). This is in satisfactory agreement with the estimated area of the dust bands. As we argue that the prominent asteroid families may provide the emitting area observed by IRAS, then the area of the entire asteroid belt might be expected to dominate the IRAS zodiacal signal. Our calculation of cross-sectional area, however, may not be applicable to the entire asteroid belt. Because the large asteroids are probably gravitationally-bound "rubble-piles"²⁴, they do not contribute to the population of small collision products. Accordingly, although the total volume of the prominent Hirayama families is only a few per cent of the total volume of the asteroid belt, the debris derived from the original catastrophic breakup of the parent asteroids or from the gradual

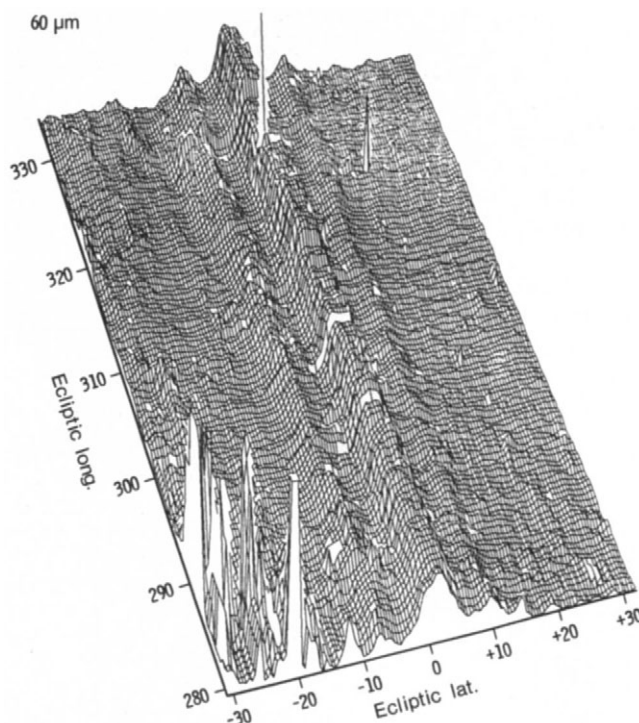


Fig. 1 IRAS data at a wavelength of $60 \mu\text{m}$ taken from scans across the ecliptic plane. The data are spatially averaged over 0.5° and the much stronger but smoother zodiacal contribution has been removed. Three major dust bands are found in, and on either side of, the ecliptic. From ref. 20.

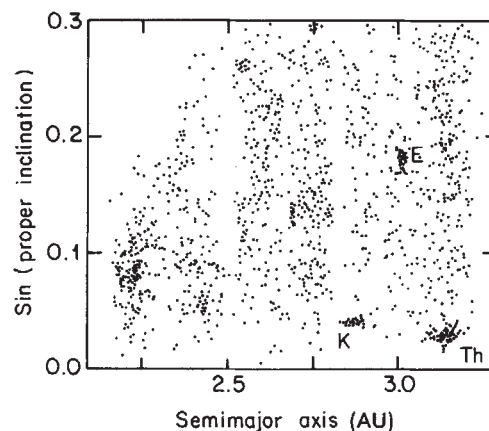


Fig. 2 The sine of the proper inclination and the semimajor axis of all asteroids listed in the TRIAD file, after ref. 34. The three most prominent families, Koronis (K), Eos (E) and Themis (Th) are identified.

Table 2 Size distributions of Hirayama asteroid families

Family	Albedo	R_e (km)	$r_{\max}$ (km)	r_0 (km)	A^* (cm^2)	$A^\dagger$ (cm^2)
Koronis	0.17 ± 0.05	43	23	37	2.0×10^{18}	6×10^{17}
Eos	0.09 ± 0.04	90	53	75	1.2×10^{19}	4×10^{18}
Themis	0.07 ± 0.03	124	99	98	2.3×10^{19}	7×10^{18}
Total					4×10^{19}	1×10^{19}

* $r_{\min} = 100 \mu\text{m}$.

† $r_{\min} = 1 \text{ mm}$.

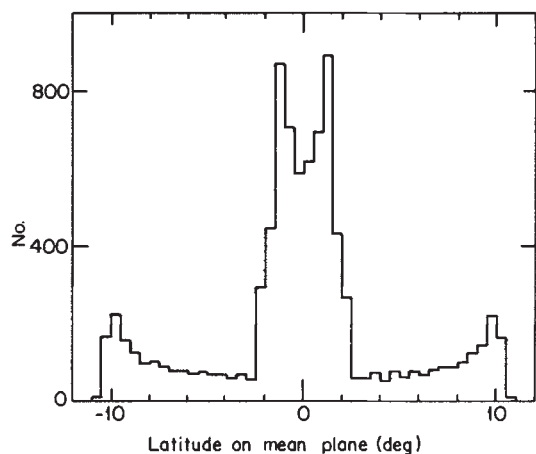


Fig. 3 The latitudinal distribution (integrated over all longitudes) of test particles spread uniformly in mean anomaly (or time) on the orbits of the known family members. 50 test particles were distributed along the orbits of each of the 42 Koronis family members, the 62 Themis family members, and the 74 Eos family members. The sum of the three distributions is shown. The latitudes are measured with respect to the mean plane of the Eos family, the orientation of which with respect to the ecliptic is determined by I_f and Ω_f (see Table 3). Because the three families have similar semimajor axes, the orientations of their mean planes are approximately the same.

commutation of the resultant family members may be much more prominent in the smaller size ranges that dominate the total cross-sectional area of the belt.

We consider that the latitudinal distribution of the prominent Hirayama families and their estimated total area make these asteroidal collision products the most likely candidate for the source of the Solar System dust bands observed by IRAS. We now describe further measurements that could be made to test this hypothesis.

Forced eccentricities and inclinations

Perturbations by Jupiter and, to a lesser extent, Saturn and the other planets result in periodic variations in the eccentricities and the inclinations of asteroidal orbits. The eccentricity vector of each orbit can be described as the vectorial sum of a free component of constant magnitude e' (the proper eccentricity) and a forced component of constant magnitude e_f , as follows:

$$e \cos \tilde{\omega} = e' \cos (\tilde{\omega}'_0 + \dot{\omega}' t) + e_f \cos \tilde{\omega}_f \quad (4)$$

$$e \sin \tilde{\omega} = e' \sin (\tilde{\omega}'_0 + \dot{\omega}' t) + e_f \sin \tilde{\omega}_f \quad (5)$$

Here, $\tilde{\omega}$ is the orbit's longitude of perihelion, $\dot{\omega}'$ is the secular apsidal precession rate due to planetary perturbations and $\tilde{\omega}_f$ is the (slowly varying) forced longitude of perihelion. The constant $\tilde{\omega}'_0$ reflects initial conditions. The inclination vector can be described analogously in terms of a proper inclination I' , and a forced inclination and nodal longitude, I_f and Ω_f . To a good approximation, the secular nodal precession rate $\dot{\Omega}' \approx -\dot{\omega}'$. Table 3 lists the values of e_f , $\tilde{\omega}_f$, I_f , and Ω_f for the three asteroid families discussed above. These quantities were calculated using the theory of Brouwer and van Woerkom^{30,31} including the effects of all the planets except Pluto, but in which terms smaller than e^2 and I^2 in the disturbing function are ignored. The errors produced by this truncation are less than 5% (J. G. Williams, personal communication). Greater accuracy could be achieved by using the higher-order of Williams³². In the low-order theory, the forced eccentricities and inclinations are independent of the free eccentricities and inclinations (assuming that these quantities are small) and depend only on the semimajor axis, a , of the orbit. Hence, measurements of the distortions of the

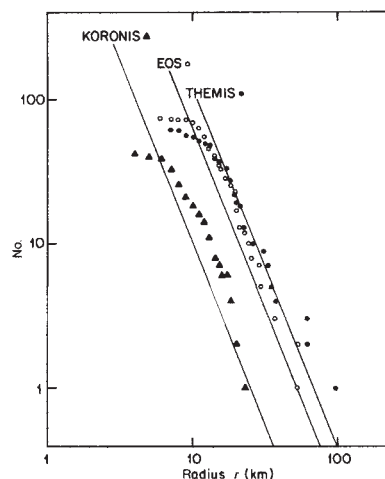


Fig. 4 Size-frequency distributions of the three prominent Hirayama families. N is the cumulative number of asteroids with radii $> r$. The intercepts of the theoretical straight-line fits are derived from the total volumes of the asteroids in each family. The slopes are $-3(q-1)$, where $q = 1.837$.

dust bands associated with the forced orbital elements may enable the distances of the bands to be deduced.

Geometrically, the forced inclination implies that all orbits with a particular value of semimajor axis precess about a common mean plane, whose inclination and ascending node are given by I_f and Ω_f , respectively. (This mean plane is close to, but not coincident with, the Solar System's invariable plane.) The peaks in the latitudinal distribution of an asteroid family (see Fig. 3) are symmetrically disposed with respect to this mean plane and, in a Sun-centred coordinate system, form two parallel bands around the sky separated by $2I'$. Variations in the apparent latitudinal separation of the bands as seen from the Earth,

Table 3 Orientation of forced eccentricities and inclinations with respect to the ecliptic

Family	e_f	$\tilde{\omega}_f$ (deg)	I_f (deg)	Ω_f (deg)
Koronis	0.0371	6.2	1.16	96.1
Eos	0.0373	7.6	1.19	97.1
Themis	0.0379	8.7	1.22	97.8

corresponding to changes in telescope pointing, also give a method of determining the distances of the bands³³. In this method, however, allowance must be made for the variation associated with the forced eccentricity, discussed below.

The orientation of the mean plane with respect to some reference plane (the forced inclination, I_f) and the longitude of the ascending node (Ω_f) are diagnostic of the semimajor axis a . Measured with respect to the Earth ecliptic and the equinox of 1950.0 (for observational purposes, a more convenient reference than the invariable plane), I_f increases from a minimum of 0.60° at 2.185 AU to 1.23° at 3.2 AU, while Ω_f increases from 315.2° at 2.0 AU to 98.0° at 3.2 AU (ref. 30). The appearance of the putative Themis band in a Sun-centred ecliptic coordinate system is shown in Fig. 5A. The sinusoidal variation of mean latitude with longitude has an amplitude of I_f and the phase of the variation is determined by Ω_f .

The effects of a forced eccentricity on the spatial distribution of an asteroid family or a dust band are analogous to those produced by a forced inclination and are illustrated for the

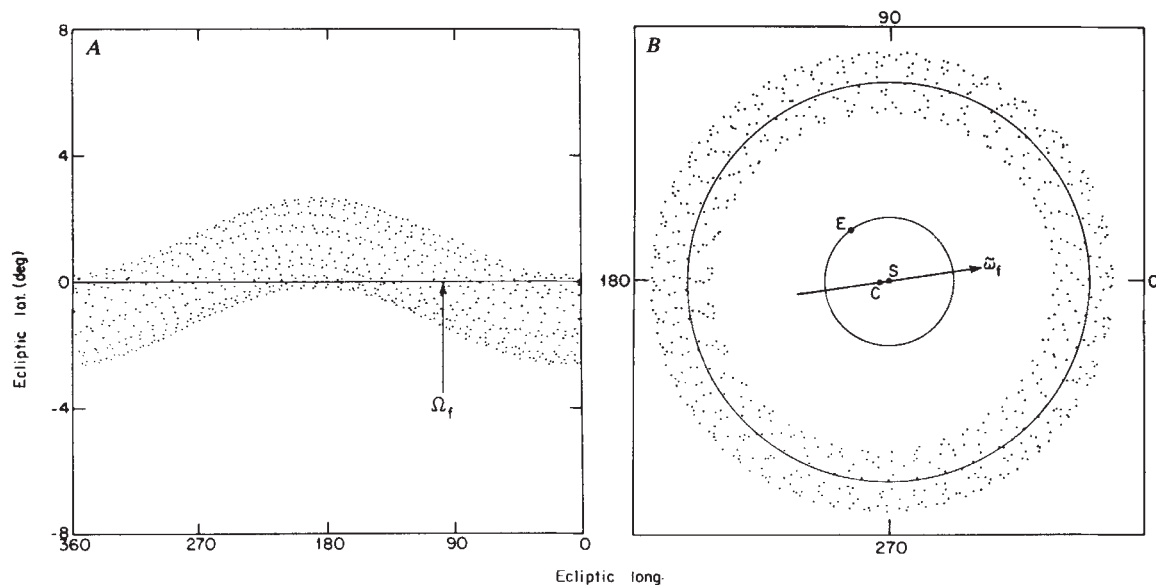


Fig. 5 Two views of the putative Themis dust band. The latitudinal width of the band in *A*, in a Sun-centred coordinate system, is $21'$ at all longitudes. The inner and outer envelopes of the particle orbits in *B* are circularly symmetric about a point *C* which is displaced from the Sun *S*, by a distance ae_f , in a direction opposite to that of the longitude of perihelion of the forced eccentricity, $\tilde{\omega}_f$. The radial width of the circular dust band is $2ae_f$. The solid circle within the dust band is centred on *S* and has a radius equal to the mean semimajor axis, *A*, of the Themis family. The inner solid circle denotes the orbit of the Earth *E*. The striations in *A* are an artefact of the few points used in the plot and disappear as the number of points increases.

Themis band in Fig. 5*B*. The band is elliptical with an eccentricity e_f and a longitude of pericentre $\tilde{\omega}_f$; its radial width is $2ae_f$. To first order in e_f , the inner and outer envelopes of the band are circles centred on a point *C*, displaced from the Sun by an amount ae_f in a direction opposite to that of $\tilde{\omega}_f$. The angular number density of the dust particles varies as

$$1 - 2e_f \cos(\lambda - \tilde{\omega}_f) \quad (6)$$

where λ is the ecliptic longitude, whereas the mean distance of the band from the Sun varies as

$$1 - e_f \cos(\lambda - \tilde{\omega}_f) \quad (7)$$

Thus, the number density of particles is a minimum in the direction of $\tilde{\omega}_f$, but at $\lambda = \tilde{\omega}_f$ the particles are both closer to the Earth's orbit and hotter. These effects more than compensate for the lower particle number density, so that the dust band is predicted to be brightest at pericentre.

Figure 6 shows the expected variation with ecliptic longitude of the thermal flux at the four wavelengths that correspond to the IRAS windows. This particular calculation applies to the Themis band, but calculations for Eos and Koronis give almost identical results. For this heuristic model, we have assumed (1) that the dust particles have an emissivity of 0.9, a geometric albedo of 0.1 and a phase integral of 0.6; (2) that the Earth's orbit is circular; and (3) that the pointing direction of the IRAS telescope is always tangential to the Earth's orbit. The mean temperature of dust particles associated with the Themis family is 160 K. At this temperature, the Planck function peaks at a wavelength of $\lambda = 18 \mu\text{m}$. At shorter wavelengths, the modest temperature change between aphelion and perihelion (~ 25 K) produces a comparatively large change in thermal flux and thus the longitudinal variation in flux is predicted to be strongest in the $12\text{-}\mu\text{m}$ window.

The phase of the longitudinal variation in brightness is determined by $\tilde{\omega}_f$ and is, therefore, diagnostic of *a*. The variations of e_f and $\tilde{\omega}_f$ with *a* are complex due to the existence of secular resonances at 2.03 AU (the S_6 resonance) and at 2.64 AU (the S_{10} resonance)³⁰. Ignoring the very large changes that occur close to these resonant locations, however, we conclude that between 2.2 and 3.2 AU, e_f generally decreases from ~ 0.054 to ~ 0.035 and then gradually increases to ~ 0.038 , whereas $\tilde{\omega}_f$

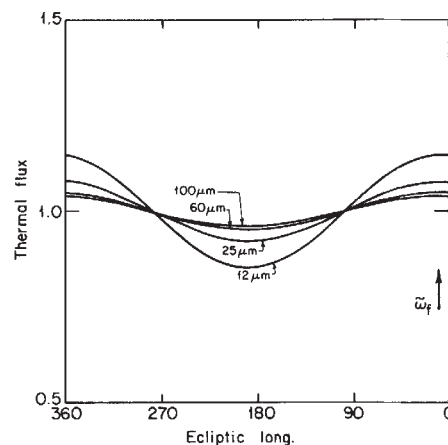


Fig. 6 Expected variation with Sun-centred ecliptic longitude of the thermal radiation from dust particles associated with the Themis family at wavelengths of 12, 25, 60 and $100 \mu\text{m}$. The arrow marks the longitude of perihelion of the forced eccentricity, $\tilde{\omega}_f$. The longitude refers to the heliocentric longitude of the dust particles, rather than to the observed geocentric longitude, although the flux is that observed at the Earth.

increases from 333.8° to 9.2° . The amplitude of the brightness variation is largely determined by e_f and is thus also diagnostic of *a*. The radial width of the dust band is determined by e_f , and will affect both the apparent, that is, the flux-weighted, mean radius of the band and the mean colour temperature derived from brightness measurements in the four IRAS wavelength channels.

Conclusions

A detailed analysis of the IRAS data should yield information on all the orbital elements of the dust particles and hence should disclose the origin of the dust bands. We have shown here that the orientation of the mean plane of a band (or pair of bands) is determined by the forced inclination and nodal longitude of the particles' orbits, whereas the forced eccentricity and peri-

centre longitude lead to predictable longitudinal brightness variations. Each of these four quantities depends in turn on the semimajor axis, as long as e and I remain small. The angular separation of a pair of bands, as viewed from the Sun, provides a direct measurement of the proper inclination, I' . The remaining orbital element of interest, the proper eccentricity, e' , may be derivable from a careful study of the apparent mean radius and/or colour temperature of a band. These results are applicable to, and provide a means of testing, any specific dynamical model of the dust bands. Apart from the asteroid family model

presented here, the most plausible alternative model would involve the disintegration of two or more comets. In this case, however, the small- e approximations would require some revision; nevertheless, our approach should be equally suitable.

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Mechanism of colour discrimination by a bacterial sensory rhodopsin

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A photosensitive protein resembling the visual pigments of invertebrates enables phototactic archaebacteria to distinguish colour. This protein exists in two spectrally-distinct forms, one of which is a transient photoproduct of the other and each of which undergoes photochemical reactions controlling the cell's swimming behaviour. Activation of a single pigment molecule in the cell is sufficient to signal the flagellar motor. This signal-transduction mechanism makes evident a colour-sensing capability inherent in the retinal/protein chromophore.

MANY bacterial species alter their swimming behaviour in response to environmental stimuli. Chemotactic bacteria, for example, sense particular nutritive and noxious substances and respond to them as attractants and repellents, respectively¹. *Halobacterium halobium*, a flagellated archaebacterium which grows in saturated brine, exhibits chemotaxis and is also phototactic; the cells are attracted to long wavelength visible light (the 'red' light response) and repelled by shorter wavelength light (the 'blue' light response)². The first quantitative studies of these responses² revealed both sensitivities present simultaneously. In the absence of photostimuli, the cells move in a three-dimensional random walk, swimming short stretches in an approximately straight line and periodically reversing direction through roughly 180°. Light intensity gradients bias the random walk by modulating the frequency of reversals³. Increases in blue or decreases in red light intensity are interpreted by the cell as movement in an unfavourable direction causing reversal of swimming direction. The opposite intensity changes suppress reversals. As in the case of bacterial chemotaxis, the flagellar motor responses are transient; the cells adapt to the new light intensity and resume their prestimulus reversal frequency³.

Vitamin A aldehyde (retinal) forms the chromophore of visual

pigments and is required also for photosensory reception in halobacterial phototaxis^{4,5}. Two retinal-containing pigments, bacteriorhodopsin and halorhodopsin, function as light-driven ion transporters in *H. halobium*⁶. These pigments may be attractant receptors and their short wavelength photointermediates repellent receptors⁷. Mutants lacking both bacteriorhodopsin and halorhodopsin exhibit normal colour-discriminating phototaxis⁸, indicating that at least one additional retinal pigment must exist in this organism.

We have detected this third photoreactive retinal-containing membrane protein in *H. halobium*⁹. Like bacteriorhodopsin and halorhodopsin, photoexcitation of the third pigment is followed by a sequence of thermal steps returning the molecule to its original state. This cyclic process (or photocycle) has a half-time of ~0.8 s at 23 °C, in contrast to the faster (~10 ms) photocycles of bacteriorhodopsin and halorhodopsin. The slow-cycling rhodopsin does not appear to mediate active ion transport^{8,9} and is the only spectroscopically detected retinal pigment in phototactic strains which lack bacteriorhodopsin and halorhodopsin^{9,10}. Addition of retinal to a mutant blocked in retinal synthesis generates both slow-cycling rhodopsin and phototaxis with similar kinetics and retinal concentration dependence^{10,11}. These properties and its absorbance in the region of attractant

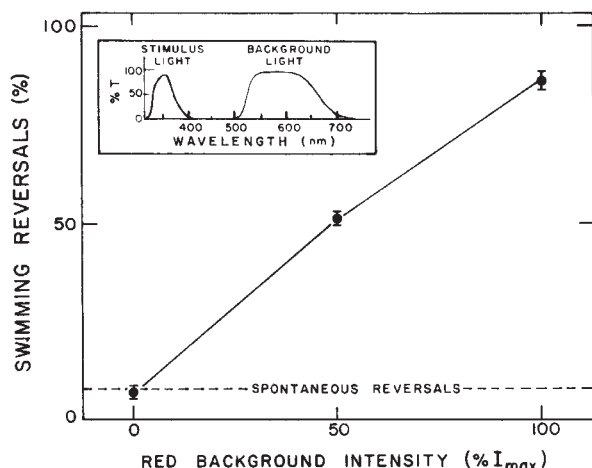


Fig. 1 Dependence of near ultraviolet repellent response on visible background light. The figure shows the % of cells reversing within 2 s of the near UV stimulus as a function of red background intensity. Points are average values $\pm$ extreme values of 2 separate determinations, each based on >40 cells. The inset shows the transmittance spectra of the filter combinations used for stimulus and visible background irradiation. The stimulus and observation light from a 150 W mercury (Hg) lamp and 50 W tungsten Zeiss microscope illuminator, respectively, were combined through a glass beam splitter which allowed passage of 30% of the infrared observation light (Kodak 87A filter) and 30% of the Hg lamp radiation. The combined beam illuminated the cells through the microscope phase condenser. The red background light was provided by an optically collimated 50 W tungsten light source reflected by a quartz beam-splitter mounted below the condenser. Light fluxes at the sample were measured with a Kettering 65 Radiometer and intensities calculated using irradiation areas measured with a calibrated ocular Zeiss micrometer. Light intensity was varied with Kodak neutral density filters. Stimulus light intensity, $1,400 \text{ erg cm}^{-2} \text{ s}^{-1}$. Maximum red background intensity (I_{max}), $7.2 \times 10^4 \text{ erg cm}^{-2} \text{ s}^{-1}$. Infrared radiation, $>750 \text{ nm}$.

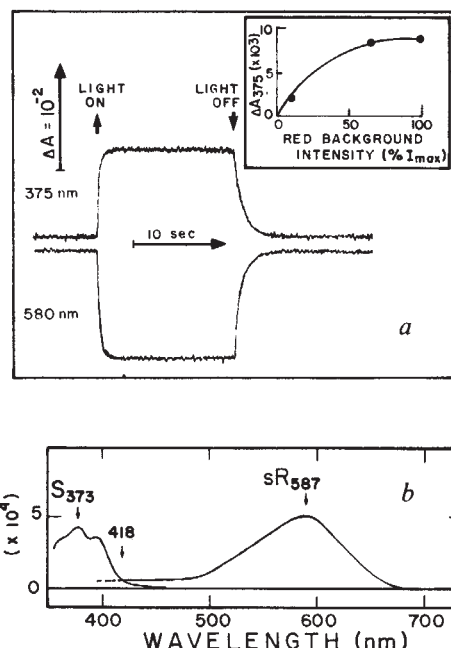


Fig. 2 *a*, Absorbance changes of sensory rhodopsin (sR) at 375 and 580 nm induced by red background light. The sample was a suspension of Flx3R vesicles, prepared vesicles, prepared and regenerated in a $3 \times 3 \text{ mm}$ quartz cuvette clear on four sides with all-*trans* retinal as described¹⁰. The regeneration of sensory rhodopsin was monitored as described¹⁰, use of the regenerated system permitting precise quantitation of the sensory rhodopsin content. Flx3 vesicles containing native sensory rhodopsin gave the same phototransients as the regenerated protein in Flx3R shown here. Flash-induced absorbance changes were measured as described⁹. Background excitation was provided by a tungsten halogen lamp beam passed through a long pass Corning 2-61 (cutoff 610 nm) and Dittic Optics 700 nm short pass filter combination and applied to the sample at 90° with respect to the measuring beam. The maximum red light intensity (I_{max}) was $4.25 \times 10^5 \text{ erg cm}^{-2} \text{ s}^{-1}$. The inset shows the light intensity-dependence of the absorbance changes at 375 nm (ΔA_{375}). *b*, Spectrum of S_{373} and sR_{587} . The latter is the spectrum obtained by regeneration of sensory rhodopsin apoprotein with all-*trans* retinal⁹ and the former was calculated by adding the sR_{587} spectrum to the light-induced difference spectrum obtained using the system described in *a*. The extinction coefficients are based on our measurements^{9,10} and have absolute uncertainty of $\pm 15\%$.

(red) sensitivity¹⁰ make this protein a likely candidate for the attractant photoreceptor. The repellent photoreceptor, however, poses a problem because no separate pigment active in the region of repellent (blue) sensitivity is detectable by sensitive flash photolysis analysis (detection limit 50 molecules per cell, unpublished data). The work we report here shows that a solution to this dilemma can be found within the slow-cycling rhodopsin photoreactions. The repellent receptor is expected to absorb maximally near 370 nm according to phototaxis action spectra^{2,4}; photoexcitation generates an intermediate species with maximal absorbance at 373 nm (S_{373}) within a few microseconds⁹ which, in addition to its slow thermal relaxation, can more rapidly reconvert to the red-absorbing form of the molecule (sR_{587}) by absorption of a second photon⁹.

We considered that slow-cycling rhodopsin in its S_{373} form might mediate the repellent responses. If this is correct, no repellent responses should occur in the absence of S_{373} and repellent sensitivity should appear when S_{373} is generated by illumination of sR_{587} . The work presented here shows these predictions are borne out and reveal a mechanism for colour-sensing based on the dual role of slow-cycling rhodopsin as attractant and repellent photosensory receptor.

Swimming behaviour

The absorption spectrum of sR_{587} is so broad that any visible light will generate S_{373} . Thus all published measurements of repellent responses have been made in the presence of S_{373} generated by the light used to observe the bacteria. To avoid exciting sR_{587} , we here observe the cells with light of wavelengths $>750 \text{ nm}$ using an infrared-sensitive camera and track the cells on a video monitor. This light is beyond the absorption range of sR_{587} and therefore no S_{373} is generated. Under these conditions, strong UV light which has been found previously¹² to be

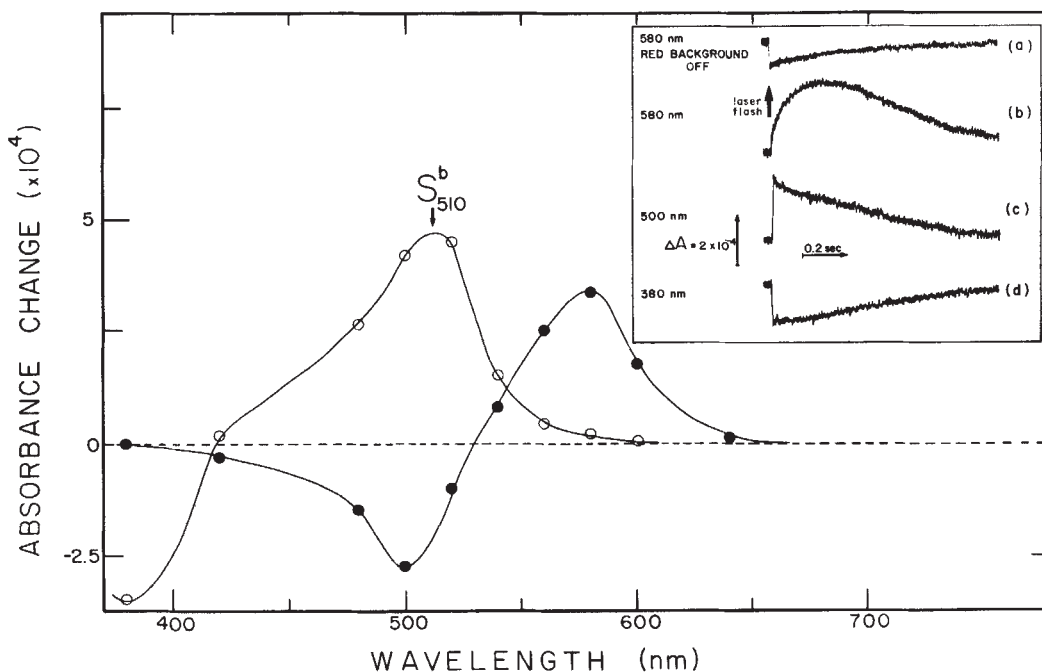
strongly repellent, causes no change in reversal probability (Fig. 1). Furthermore, addition of red background light which generates S_{373} sensitizes the cells to the repellent stimulus, causing nearly every cell to reverse swimming direction within 2 s (Fig. 1). We attribute this sensitization to the generation of a photostationary state containing the repellent receptor (S_{373}) by red light.

One might argue that the adaptation to the red stimulus lowered the threshold sensitivity to blue light because the background red light generated a transient attractant response when it was turned on. This possibility is ruled out because we show here that red light sensitizes the blue response even if it is delivered simultaneously with the blue stimulus.

Photochemical reactions

Because of its fast rise and slow decay, S_{373} accumulates in significant amounts in natural light absorbed by sR_{587} , producing a photostationary state mixture of the two species sR_{587} and S_{373} . The absorption spectra of these species, calculated from the photostationary state difference spectrum, are shown in Fig. 2. The spectra are sufficiently broad to permit laser photoexcitation of both species at 406 nm so that flash-induced absorbance changes at their regions of maximum absorption could be monitored with minimal interference from the intense actinic flash.

Fig. 3 Flash-induced absorbance changes of S_{373} . Inset, transient absorption changes of the sample shown in Fig. 2 in the absence (a) and in the presence (b–d) of 100% I_{max} red background illumination at three selected wavelengths. Actinic laser flash: 406 nm, 10 ns, 0.4 mJ. Main figure from traces shown in the inset and at other wavelengths the difference in the amplitude of absorbance change between 0 and 100 μs ($\circ$) and between 200 ms and 100 μs ($\bullet$) were calculated and plotted as functions of wavelength. Note that at 580 nm the ratio of the forward to back reaction absorbance change amplitude is the same as the ratio of absolute absorbances of sR_{587} and S_{373} at 406 nm (Fig. 2). This indicates that the photochemical quantum yields of the two reactions are nearly equal.



In the absence of background illumination, excitation at 406 nm causes a transient depletion of sR_{587} (Fig. 3a). This is due to the sR_{587} to S_{373} photoconversion as shown previously⁹. In the presence of saturating background illumination, which depletes the sample of sR_{587} (Fig. 2a), excitation causes a transient regeneration of sR_{587} from S_{373} (Fig. 3b, d). Absorbance changes measured at 500 nm (Fig. 3c) reveal an intermediate state in the S_{373} to sR_{587} reaction which forms in less than 50 μs . Further analysis of flash kinetic data at other wavelengths (Fig. 3) shows that this intermediate state absorbs maximally near 510 nm and decays into sR_{587} with a half-time of about 80 ms. Absorption of red background light by sR_{587} regenerates S_{373} (Fig. 3d). Note that this establishes a cyclic reaction containing two photon absorption steps (Fig. 4).

Colour-sensing mechanism

Spectral discrimination by slow-cycling rhodopsin can be understood as an interplay between the two-photon cycle discussed above and the one-photon cycle generated by photoexcitation of sR_{587} and its subsequent thermal steps (Fig. 4). We propose that the one-photon reaction generates an attractant signal, transmitted to the flagellar motor by an attractant signal transduction pathway. Increases (decreases) in this signal suppress (induce) flagellar reversals. These effects occur in the absence of any background illumination. In contrast, the two-photon cycle generates a repellent signal, transmitted and amplified separately from the attractant signal, but integrated eventually before or at the site of action on the flagellar motor³. Increases (decreases) in this signal induce (suppress) flagellar reversals.

Test of the mechanism

Previous studies have shown that the cells are at least an order of magnitude more sensitive to repellent (blue) light than to attractant (red) light intensity changes^{2,3}. According to our interpretation this sensitivity difference must be conferred by amplification step(s) beyond the photoreceptor, because there can never be more repellent receptor molecules (S_{373} than the maximum number of molecules available for attractant reception (sR_{587}) and their photochemical quantum yields are nearly equal (see Fig. 3 legend). Therefore the repellent signal from the two-photon cycle must be at least an order of magnitude more effective in inducing reversals than the one-photon cycle is in suppressing them. This leads to a seemingly paradoxical predic-

tion which is a strong test of the mechanism: we should be able to construct conditions in which an increase in red light will generate a repellent rather than the usual attractant response.

According to the proposed mechanism (Fig. 4), the repellent signal produced by S_{373} photoexcitation should be generated by any of three illumination patterns. First, photoexciting S_{373} previously generated by background red light (as in Fig. 1). This is the usual stimulus which makes blue light a repellent under natural illumination.

Second, imposing an intense background of blue illumination which, in the absence of red light, will not influence the cells' swimming behaviour because slow-cycling rhodopsin is almost completely in the sR_{587} form, followed by stimulation with red light. The S_{373} thereby generated will undergo the two-photon cycle, which should cause the repellent rather than the attractant response. This condition is realized in Fig. 5 where a given increase in red light was delivered in the absence and in the presence of blue background illumination. A clear suppression

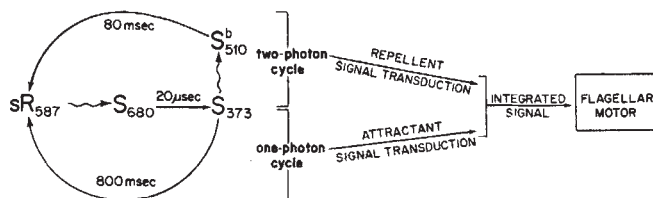


Fig. 4 Mechanism of colour discrimination in *H. halobium* phototaxis. Photon absorption by sR_{587} , the dark-adapted form of sensory rhodopsin, generates a short-lived species S_{680} which rapidly decays to S_{373} . The S_{373} form can return to the sR_{587} form by either of two paths: a purely thermal relaxation or a photoinduced path. If S_{373} does not absorb a photon, it relaxes thermally to sR_{587} completing the 'one-photon' photocycle. During its transit through the one-photon cycle, the sensory rhodopsin molecule generates an attractant signal to the flagellar motor. If S_{373} absorbs a photon, it generates the intermediate S_{510}^b . The relaxation of S_{510}^b to sR_{587} completes a cycle containing two photon absorption steps. After absorption of the second photon in the 'two-photon' cycle, sensory rhodopsin generates a repellent signal to the flagellar motor. The attractant and repellent signals are integrated prior to or at their site of action on the flagellar motor³.

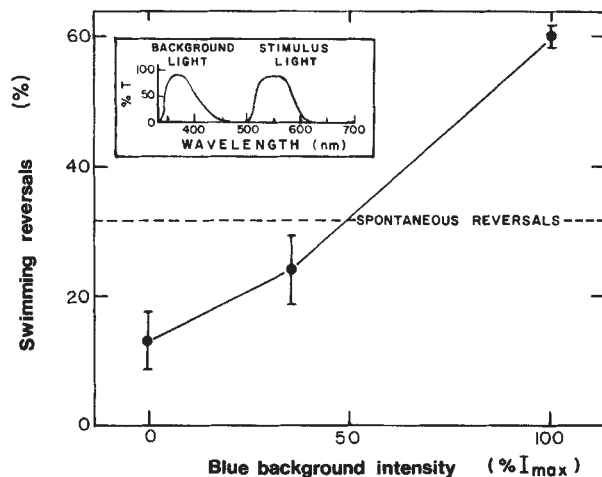


Fig. 5 Effect of near ultraviolet background light on cell behavioural response to red light. Experimental conditions as described in Fig. 1 except that the response was assayed as the % reversals within 3 s of a red light stimulus. The range of spontaneous reversal frequencies of populations used in these studies was $14\% \pm 7\%$ reversing within 2 s. All effects of background illumination on behaviour reported here and in Figs 2 and 6 have been confirmed in populations with both high and low spontaneous reversal frequencies.

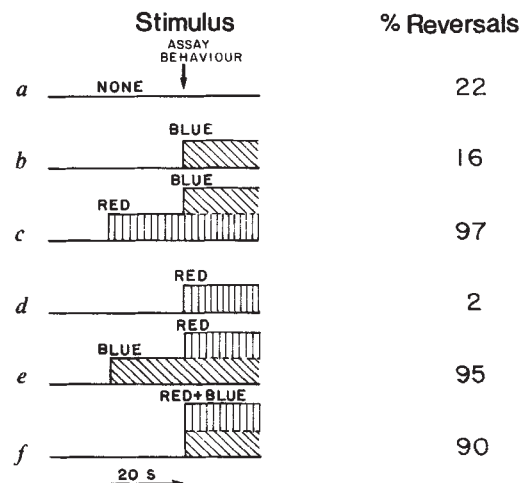


Fig. 6 Behavioural response under various permutations of stimulus and background light. Values are averages of duplicate measurements which vary by less than 15%. Experimental conditions as described in Fig. 1 except that reversals were assayed within 3 s of the stimulus, blue and red light were delivered using a tungsten light source and wavelengths were selected by adding or removing filters from the single beam. The Hg light source was blocked in this experiment. 'Blue', 380–433 nm (Optics Technology 433 short pass filter + 2 mm Plexiglass + two 1–75 Corning infrared absorbing glass), intensity $3.3 \times 10^4 \text{ erg cm}^{-2} \text{ s}^{-1}$. 'Red', 530–700 nm (Corning 3–68 + two Corning 1–75), intensity $1.0 \times 10^5 \text{ erg cm}^{-2} \text{ s}^{-1}$. 'Red + Blue', The tungsten beam filtered through two Corning 1–75 + 2 mm Plexiglass, intensity $1.5 \times 10^5 \text{ erg cm}^{-2} \text{ s}^{-1}$.

of spontaneous reversals occurs in the absence of background blue as expected from the one-photon cycle attractant signal. The dramatic result is that the same red light stimulus elicits a repellent (reversal-inducing) response in the presence of the highest blue light intensity.

Third, delivering both red and blue lights simultaneously at the same intensities as Fig. 5. Again the repellent response should result since it makes no difference whether the blue light was present prior to the generation of S_{373} .

All the permutations of blue and red light stimuli are shown in Fig. 6. Our results can be interpreted in terms of the proposed model. Figure 6a gives the spontaneous reversal frequency of 22%, which is not affected significantly by blue alone. Figure 6b shows 16% reversals, because there is essentially no S_{373} (some S_{373} will be generated by the weak sR_{587} absorption in the blue, but calculations show the amount of S_{373} formed is so little that no two-photon cycling will occur in the time of the measurement). The three illumination patterns discussed above are shown by Fig. 6c, e and f respectively, all inducing an almost complete reversal response. Red light alone significantly suppresses reversals (Fig. 6d).

Conclusions

The experiments we reported previously strongly implicated slow-cycling rhodopsin as the attractant receptor for *H. halobium* phototaxis^{9,10}. The evidence presented here implicates this protein as the repellent receptor as well. We prove here that, like S_{373} , the repellent receptor is a photoproduct of a slow-cycling, red-absorbing, retinal pigment with the properties of sR_{587} .

From these experiments we propose a mechanism in which slow-cycling rhodopsin has two signal transducing forms, one transmitting an attractant signal and the other a repellent signal to the flagellar motor (Fig. 4). Increases in the repellent signal or decreases in the attractant signal induce flagellar reversals. Conversely, decreases in the repellent signal or increases in the attractant signal suppress flagellar reversals. Both induction and suppression of reversals are transient responses, demonstrating adaptation of the cell to the signals. The net effect is that the motility apparatus of the cell responds to changes in the numbers of slow-cycling rhodopsin molecules undergoing the one-photon and two-photon cycles. In view of these results, we suggest the original name⁹ 'slow-cycling rhodopsinlike pigment' be changed

to 'sensory rhodopsin' of *H. halobium*, reflecting more clearly the physiological function of the molecule.

The absorption spectrum of S_{373} matches closely the $\sim 370 \text{ nm}$ action spectrum peak reported for phototaxis repulsion^{2,4,5}. However, the absorption spectrum of sR_{587} matches some but not all of the published action spectra for phototaxis attraction. The attractant response was reported to have a single peak at $\sim 565 \text{ nm}$ in wild-type^{2,5} and two peaks, one at $\sim 565 \text{ nm}$ and the other at $\sim 590 \text{ nm}$, near the sR_{587} absorption maximum in a bacteriorhodopsin-deficient strain¹². The disagreement may be caused by the presence of bacteriorhodopsin and halorhodopsin which may influence the motility response directly or indirectly.

Dencher¹³ and Hildebrand and Schimz¹⁴ have proposed that two distinct receptors mediate the attractant and repellent sensitivities. Our data prove that the retinal-dependent repellent receptor is a photoproduct of a red absorbing pigment. Therefore their proposal requires the existence of at least one additional hypothetical red-absorbing retinal pigment. Our model requires only the single identified slow-cycling rhodopsin and, moreover, makes several specific quantitative predictions: (1) a delayed blue flash following a red flash will change from an attractant to a repellent stimulus with delay time predicted by the $20 \mu\text{s}$ rise time of S_{373} ; (2) mutants deficient in slow-cycling rhodopsin would be predicted to lose both attractant and repellent photosensitivities, and reversion to normal phototaxis behaviour should be accompanied by return of the pigment.

A remarkable finding from our measurements is that photoexcitation of a single molecule of S_{373} appears to be sufficient to generate a reversal-inducing repellent signal to the flagellar motor. From the measured intensity ($3.6 \times 10^4 \text{ erg cm}^{-2} \text{ s}^{-1}$) and spectrum of the background 'red' illumination (Fig. 1), the sR_{587} and S_{373} molar extinction spectra (Fig. 2b) and the parameters of the photocycle (Fig. 4), we calculate 2% of the sensory rhodopsin molecules will be in the S_{373} form in the photostationary state at 50% maximal intensity for red background (Fig. 1). From the regeneration of sensory rhodopsin absorption by retinal (see Fig. 2 legend) and the sensory rhodopsin extinction ($54,000 \text{ M}^{-1} \text{ cm}^{-1}$) at that wavelength, we measure 5×10^3

molecules per cell, ~ 100 of which therefore will be in the S_{373} form. From the quantum flux of blue light used for the stimulus (Fig. 1) and the measured S_{373} extinction in the stimulus spectral range (Fig. 2b), we calculate the S_{373} population in the average cell will absorb only about two photons in the 2-s period over which reversals were assessed. Taking the quantum efficiency for photochemical reaction to be 0.5 ± 0.2 , covering the range of all known retinal pigments, only $\sim 0.7\text{--}1.4$ S_{373} molecules per cell on average would be activated photochemically by a stimulus which causes over 50% of the cells to respond. Therefore photoexcitation of a single S_{373} molecule appears to be sufficient to alter the cell's swimming behaviour.

The cell is at least an order of magnitude less sensitive to attractant than repellent light^{2,3}. In our interpretation this means the amplification of the S_{373} signal by the signal transduction machinery (Fig. 4) is at least $10\times$ greater than that of sR_{587} . The relative amplification in the two signalling pathways may depend on the specific conditions and stage of cell culture growth (unpublished observations).

The colour-sensing mechanism described here depends on the existence of two spectrally-distinct signalling forms of the same receptor. This could be a unique property of the sensory rhodopsin of halobacteria. Alternatively, the sensory rhodopsin mechanism may reflect an inherent potential of retinal/protein

complexes—photoreversibility of photoconversion products of the retinal/opsin chromophore—important to the evolution of the rhodopsin family as photosensory receptors. Even retinal pigment photoproducts which are too short-lived to accumulate under physiological conditions (such as the K, L and M intermediates formed from bacteriorhodopsin⁶ and the early photoproducts formed from mammalian rod rhodopsin¹⁴) are photoreversible. Invertebrate rhodopsins, like sensory rhodopsin and the non-retinal plant chromoprotein phytochrome, are photochromic; that is, they have photoreactions with thermal relaxation sufficiently slow to cause significant photostationary state accumulation of their photoreversible conformations¹⁶. The sensory rhodopsin mechanism, preserved in the archaebacterium *H. halobium*, may have provided a primitive colour-sensing capability. In the evolution of visual systems, this mechanism may have been maintained in some organisms but replaced in others by multiple receptor mechanisms with increased colour resolution.

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The *neu* oncogene: an *erb-B*-related gene encoding a 185,000- M_r tumour antigen

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A series of rat neuro/glioblastomas all contain the same transforming gene (neu) which induces synthesis of a tumour antigen of relative molecular mass (M_r) 185,000 (p185). The neu oncogene bears homology to erb-B and the tumour antigen, p185, is serologically related to the epidermal growth factor (EGF) receptor. The two proteins, EGF receptor and p185 appear to be distinct, as they coexist in nontransformed Rat-1 cells.

GENE transfer (transfection) has revealed the presence of oncogenes in the genomes of a variety of spontaneous and chemically induced tumours. Many of these oncogenes are allelic versions of one of the three genes belonging to the *ras* gene family. In several cases, the functional differences between these oncogenes and their cognate proto-oncogenes derive from single nucleotide changes, mutations which apparently have occurred in the target tissue as one of the steps of carcinogenesis (reviewed in refs 1, 2).

We have been investigating a family of transforming genes in the genomes of several rat neuro/glioblastoma cell lines derived from tumours induced by ethylnitrosourea (ENU)³. Injection of pregnant BDIX rats with this carcinogen during day 15 of gestation results in the appearance of neuro/glioblastomas in

the offspring. DNA prepared from four neuro/glioblastoma cell lines transforms NIH 3T3 cells when introduced by transfection⁴, but Southern blot analysis of the genomes of the transformed cells gave no indication of the presence of acquired *ras* oncogenes (A.L.S. and L.V., unpublished results), suggesting that a *ras* gene was not involved in the transforming activity.

The four independently activated neuro/glioblastoma oncogenes exhibited a common pattern of sensitivity or resistance to inactivation by restriction enzyme cleavage (C. Shih and R.A.W., unpublished data), suggesting that all are allelic versions of a common antecedent gene. We have applied the term '*neu*' to this group of apparently related genes.

These neuro/glioblastoma oncogenes are associated with the expression of a common tumour antigen⁵. NIH 3T3 cells

transformed by *neu* oncogenes were used to seed tumours in young mice, the sera from which precipitated a phosphoprotein of relative molecular mass (M_r) 185,000 (185K) from lysates of all NIH 3T3 lines that carry the neuro/glioblastoma oncogenes. This protein, designated p185, was detected neither in untransformed NIH 3T3 cells nor in NIH cells transformed by various *ras* oncogenes. Furthermore, when *ras*-transformed NIH 3T3 cells were used to seed tumours in mice, no serum reactivity to p185 was induced. Thus, synthesis of p185 is strongly associated with the presence of the neuro/glioblastoma oncogenes⁵.

Results from several laboratories have shown that the cellular homologues of certain oncogenes first identified in retroviral genomes are also important in the genesis of spontaneous and chemically induced tumours (reviewed in refs 1, 2). One of these retrovirus-associated oncogenes is *erb-B*, which was recently shown to be homologous with the cellular gene encoding the epidermal growth factor (EGF) receptor⁶. The EGF receptor and p185 have similar molecular weights, and they are both phosphorylated cell-surface glycoproteins (refs 7–11 and A.L.S., D.F.S. and R. Negrin, unpublished data). Thus, we suspected a possible relationship between *erb-B* and the *neu* oncogenes. We report here on the homology of the *neu* and *erb-B* genes and on the relationship of p185 to an *erb-B* gene product, the EGF receptor.

Homology with *erb-B*

Possible homology between *erb-B* and *neu* was tested by Southern blot hybridization. DNA was prepared from a panel of NIH 3T3 cells, each of which had been transformed by DNA from one of the rat neuro/glioblastoma cell lines. The DNAs were digested with endonuclease *Eco*RI before electrophoresis. Figure 1c–p shows that NIH 3T3 cells that have been transformed by the neuro/glioblastoma DNA carry an *Eco*RI fragment that reacts with an *erb-B*-specific sequence probe. The variability in size of the new *Eco*RI fragments probably results from rearrangements in sequences flanking the transforming gene which occur during transfection. Such variability has been observed previously in cells transfected with the EJ bladder carcinoma oncogene¹². An *erb-B*-homologous fragment of similar size is present in much smaller amounts in untransformed NIH 3T3 cells (Fig. 1q).

To distinguish between the rat and mouse *erb-B* homologues and to ensure that the large *Eco*RI segments shown in Fig. 1c–p

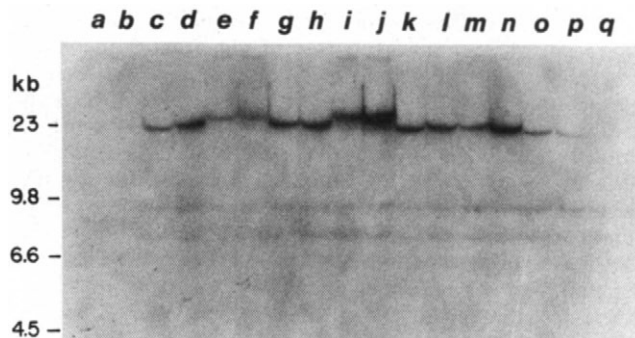


Fig. 1 Southern blot analysis of *erb-B*-related sequences in NIH 3T3 cells transformed with rat neuro/glioblastoma DNAs; *Eco*RI digestion. The DNAs analysed were obtained from the following: a, BDIX rat liver; b, B104; c, d, primary transfectants of B104 (B104-1 and B104-3); e, f, secondary transfectants of B104 (B104-1-1 and B104-1-2); g, h, primary transfectants of B103 (B103-5 and B103-1); i, j, secondary transfectants of B103 (B103-1-1 and B103-1-2); k, B50-1, a primary transfectant of B50; l, m, secondary transfectants of B50 (B50 6a-2 and B50 6a-3); n, o, primary transfectants of B82 (B82-1 and B82-3); p, B82-3-2, a secondary transfectant of B82; q, NIH 3T3. M_r standards comprising λ phage *Hind*III restriction fragments are shown on the left.

Methods: High-molecular weight DNA (10 μ g) was digested with endonuclease *Eco*RI, fractionated by electrophoresis in 0.9% agarose gels, and transferred to nitrocellulose paper. The filters were hybridized at low stringency (30% formamide, $5\times$ SSCPE, 42 °C) for 36 h with an *erb* specific probe (a *Sac*I-*Pvu*II 1.8-kb subclone of avian erythroblastosis virus²²) nick-translated with 32 P-dCTP. The filters were then washed three times at 20 °C for 10 min and for 5 h at 50 °C in $2\times$ SSCPE. The filters were exposed to Kodak X-Omat AR for 3 days at -70 °C.

are of donor rat origin, we treated the DNA of the transfected cells with endonucleases *Eco*RI and *Bam*HI before Southern blot analysis. This analysis confirmed that the transfected cells carry an acquired 4.4-kilobase(kb) *BAM*/RI DNA segment which has no counterpart in the genome of the recipient mouse cells (Fig. 2). Thus, 14 independently transfected cell lines shown to express high levels of p185 have acquired a DNA segment exhibiting some homology with the *erb-B* probe. Because these

Table 1 Immunofluorescent staining of rat and human cell lines by antibody 7.16.4

Cell lines	Description	Immunofluorescence
B104	<i>neu</i> ⁺ rat neuroblastoma	+
B82	<i>neu</i> ⁺ rat neuroblastoma	+
B50	<i>neu</i> ⁺ rat neuroblastoma	+
B15	<i>neu</i> ⁻ rat neuroblastoma	+++
B65	<i>neu</i> ⁻ rat neuroblastoma	++
B104-1-1	NIH 3T3 transfectant of <i>neu</i> ⁺ DNA	++++
NIH 3T3	Mouse fibroblast cell line	-
Rat(B104)-2	Rat-1 transfectant of <i>neu</i> ⁺ DNA	++++
Rat(B104)-3	Rat-1 transfectant of <i>neu</i> ⁺ DNA	+++
Rat(B104)-4	Rat-1 transfectant of <i>neu</i> ⁺ DNA	+++
Rat-1	Rat fibroblast cell line	+
WTCL4	Rous sarcoma virus-induced rat neuroblastoma	++
A431	Human cervical carcinoma line	-
PC3	Human prostate carcinoma line	-
MCF7	Human breast carcinoma line	-
EJ	Human bladder carcinoma line	-
G361	Human colon carcinoma line	-
HI135	Human melanoma line	-

Cell lines were prepared for FACS analysis on a Coulter Epics V Cell Sorter as described in ref. 11. Immunofluorescence was quantified by subtracting the median fluorescence channel of populations stained with FITC-conjugated rabbit anti-mouse immunoglobulin alone (negative control) from the median fluorescence channel of populations stained with monoclonal antibody 7.16.4 followed by FITC-conjugated rabbit anti-mouse (positive staining). The resulting net fluorescence channel was expressed according to the following scale: -, less than two channels; +, 5–10 channels; ++, 11–15 channels; +++, 16–20 channels; +++++, >20 channels.

cell lines derive from several independent transfections of four different neuro/glioblastoma tumour DNAs, we concluded, as we had speculated, that a common oncogene is present in the genomes of the four donor neuro/glioblastomas.

The *erb-B* oncogene probe reacts with at least two fragments produced by *Eco*RI cleavage of rat DNA (Fig. 1a, b) which are respectively 7 and 34 kb long (M.-C. Hung and A.L.S., unpublished results). The 34-kb fragment is of identical size to the rat DNA segment found in transfected NIH 3T3 cells and must represent the normal counterpart to the DNA segment bearing the *neu* oncogenes. Because *Eco*RI does not inactivate the *neu* oncogene (C. Shih and R.A.W., unpublished results), we conclude that this oncogene lies entirely within the 34-kb *Eco*RI fragment. The 7-kb *erb-B*-related DNA segment may represent a distinct but related gene. The Southern blot analysis does not indicate which of these *erb-B*-homologous segments encodes the EGF receptor, so that we could not confirm whether the *neu* gene is identical with, or only related to, the gene encoding the EGF receptor.

p185 and EGF receptor are related

The homology between *erb-B* and *neu* suggested that the EGF receptor, a known *erb-B* gene product, might be related to p185, the protein induced in cells transfected with *neu*. Two different antibody preparations were used to examine this possibility. Monoclonal antibody 7.16.4 was derived from mice immunized with B104-1-1 cells (a cell line derived by two serial passages of the *neu* oncogene through NIH 3T3 cells; ref. 4). The reactivities of the 7.16.4 antibody are indicated by the immunofluorescence data shown in Table 1. The antibody recognizes a cell-surface determinant displayed by rat neuro/glioblastomas, by a variety of normal and transformed rat cell lines and by NIH 3T3 cells transformed by the rat oncogene; it does not react with normal NIH 3T3 cells or with a variety of human tumour cell types, including the A431 cells known^{13,14} to display large amounts of EGF receptor. The 7.16.4 antibody appears to react with a rat-specific determinant of p185. Further characterization of this antibody is reported elsewhere¹¹. The second source of antibody was polyclonal anti-EGF receptor serum raised against EGF receptor purified from human A431 cells; this serum reacts with human and rodent EGF receptor¹⁵.

We used these sera to elucidate the relationship between the EGF receptor and p185. Lysates of B104-1-1 cells, metabolically labelled with ³⁵S-cysteine, were incubated with normal mouse serum, serum 7.16.4 or anti-EGF receptor serum; p185 was precipitated by the 7.16.4 serum (Fig. 3h), but not by the control serum (Fig. 3g). Incubation with anti-EGF receptor serum (Fig. 3i) yielded a protein that co-migrated with p185. This suggested that the p185 induced by the rat *neu* oncogene shares antigenic determinants with the human EGF receptor.

To confirm that the two immune sera recognize the same protein in B104-1-1 cells, labelled lysates cleared by incubation with one antibody were subsequently reacted with the other. For example, the supernatant remaining after incubation with antibody 7.16.4 was then reacted with the anti-EGF receptor serum, which did not precipitate a 185K protein from the lysate (Fig. 3l). However, anti-EGF receptor did precipitate the 185K protein from a lysate cleared with normal serum (Fig. 3k). Similarly, p185 could be precipitated by 7.16.4 serum from a lysate that had been cleared with normal serum (Fig. 3m), but not from a lysate that had been cleared with anti-EGF receptor serum (Fig. 3n). (The decreased intensity of specific and non-specific bands in lanes k to n of Fig. 3 compared with lanes g to j indicates that nonspecific losses occurred during the second round of immunoprecipitation.) These results confirm that an anti-EGF receptor serum reacts with p185. Thus, we conclude that p185 and EGF receptor have antigenic determinants in common.

Although p185 and EGF receptor are serologically related, they have different electrophoretic mobilities. The rat EGF receptor has an apparent M_r of ~170,000, while p185 is some-

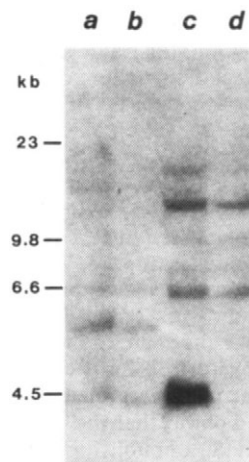


Fig. 2 Southern blot analysis of *erb-B*-related sequences; *Eco*RI plus *Bam*HI digestion. The DNAs analysed were: a, BDIX rat liver; b, B104-1; c, B104-1; d, NIH 3T3. High-molecular weight DNA (10 µg) was digested with restriction endonucleases *Eco*RI and *Bam*HI and analysed as described in Fig. 1 legend.

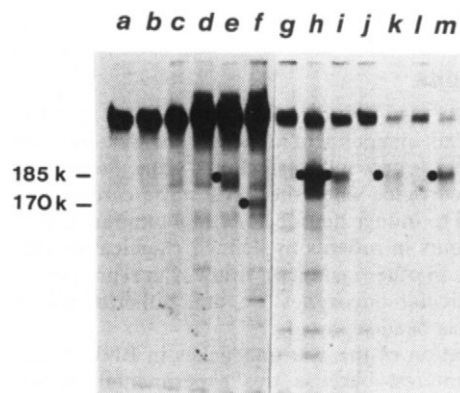


Fig. 3 Immunoprecipitation of p185 and EGF receptor. Lysates of ³⁵S-cysteine-labelled cells were incubated with antibody and subsequently precipitated. Lane a, NIH 3T3 cells incubated with normal mouse serum (NMS); b, NIH 3T3 cells incubated with antibody 7.16.4; c, NIH 3T3 cells incubated with anti-EGF receptor antiserum; d, Rat-1 cells incubated with NMS; e, Rat-1 incubated with antibody 7.16.4; f, Rat-1 incubated with anti-EGF receptor serum. g–n, B104-1-1 incubated with the following: g, NMS; h, antibody 7.16.4; i, anti-EGF receptor serum; j, NMS; k, supernatant from g, incubated with anti-EGF receptor; l, supernatant from h, incubated with anti-EGF receptor; m, supernatant from j, incubated with antibody 7.16.4; n, supernatant from i, incubated with antibody 7.16.4.

Methods: 3×10^6 cells (B104-1-1) or 3×10^5 cells (NIH 3T3 and Rat-1) were seeded in 60-mm culture dishes and labelled for 18 h in 0.8 ml of Hank's minimal essential medium containing 0.1 the usual amount of cysteine, 2% dialysed fetal calf serum, and 250 µCi ³⁵S-cysteine (977 Ci mmol⁻¹; NEN) per ml. Cells were lysed in phosphate-buffered RIPA buffer containing 1 mM ATP, 2 mM EDTA and 20 mM sodium fluoride and immunoprecipitates prepared and washed as described elsewhere²³. Portions of the lysates were incubated with NMS (1 µl), antibody 7.16.4 (concentrated, 1 µl) or 3 µl anti-EGF receptor antiserum for 60 min, then with 1 µl of sheep anti-mouse immunoglobulin (Cappel). Incubation was continued for 30 min, after which immunoprecipitates were collected using fixed *Staphylococcus aureus*. Precipitates were prepared from 300,000 cells (lanes a–f), 60,000 cells (g, h, k and l) or 30,000 cells (i, j, m and n). (Samples in g–n were all derived from the same original lysate.) Precipitates in lanes k and l were prepared from supernatants remaining after precipitation of the lysates shown in lanes g and h. Precipitates in lanes m and n were prepared from lysates first precipitated to yield the samples shown in lanes j and i, respectively. The samples were analysed by SDS-polyacrylamide gel electrophoresis in a 7.5% acrylamide-0.17% bisacrylamide gel. The gel was fluorographed²⁴ and exposed to preflashed film for 7 days at –70 °C.

what larger; this difference could arise from two sources. It is possible that the mutation(s) leading to activation of the *neu* oncogene grossly affect the structure of the encoded protein so that a gene normally encoding an EGF receptor-sized protein might, after oncogenic activation, encode a protein the size of p185. Alternatively, the *neu* oncogenes might derive from a distinct proto-oncogene that normally encodes a 185K protein.

To distinguish between these possibilities, we examined two nontransformed fibroblast cell lines, hoping to identify possible normal protein homologues of p185. We have not yet been able to identify large proteins specifically precipitated from NIH 3T3 cells by either antibody 7.16.4 or anti-EGF receptor serum (Fig. 3a-c). However, these antibodies do recognize proteins present at low levels in untransformed Rat-1 cells. The anti-EGF receptor preferentially precipitates a protein of 170K (Fig. 3f) which has a slightly greater mobility than the EGF receptor precipitated from A431 cells (D.F.S., unpublished results) and is probably the rat EGF receptor. In contrast, antibody 7.16.4 precipitates a protein (Fig. 3e) that migrates slightly more rapidly than p185 (lanes h, i), but this antibody does not precipitate the putative EGF receptor. These data indicate that the two sera recognize distinct proteins in Rat-1 cells, and support the notion that p185, induced by the *neu* oncogene, stems from a protein of similar size present in normal cells. The normal protein may be distinct from the EGF receptor.

Implications

The *neu* gene was found to be activated in cell lines derived from four of six independent rat neuro/glioblastomas, the DNAs of which were tested by transfection⁴. Thus, *neu* activation is a frequent occurrence when the ENU pulse carcinogenesis protocol is used to induce neuroectodermal tumours in rats. Induction of tumours in rodents by defined chemical or radiological regimes has, in other instances, resulted in reproducible activation of particular oncogenes^{16,17}, but, hitherto, only *ras* gene activation has been reported.

The activation of the *neu* oncogenes in ENU-treated rats is of special interest because this experimental model of carcinogenesis has been characterized in great detail. These neuro/glioblastomas are induced by exposure to ENU during a well-defined window of time in late fetal and early postnatal development. Studies by Rajewsky and co-workers indicate that proliferation of target blast cells and the absence of a DNA repair system in embryonic rat brain cells strongly influence the kinetics of tumour induction¹⁸. Moreover, these investigators have developed an *in vitro* culture system in which brain cells are explanted from fetal rats following transplacental mutagenesis with ENU. These cultures undergo a series of changes and ultimately yield tumorigenic clones with the same lag period as that required for induction of tumours *in vivo*¹⁹. By using this culture system, it may be possible to determine whether *neu* activation is an early or late event in tumorigenesis, and whether early identification of premalignant cells is possible using p185-specific antibodies.

We have found that there is nucleic acid sequence homology between *neu* and *erb-B* oncogenes, and that the EGF receptor,

encoded by an *erb-B*-related gene, is antigenically related to the *neu*-associated tumour antigen, p185. These results strongly suggest that p185 is a product of the *neu* oncogene.

The *erb-B* probe recognizes at least two DNA fragments in the rat genome, one of which encompasses a biologically active *neu* oncogene. This raises the possibility that there may be more than one functional *erb-B*-related gene in the rat genome, and that the gene which specifies EGF receptor is partially or completely separate from the *neu* proto-oncogene.

The data of Fig. 2 indicate that the anti-EGF receptor and 7.16.4 antibodies recognize distinct proteins that coexist in non-transformed Rat-1 cells. This suggests that the differences between the EGF receptor and a p185-like protein can be observed in cells that have not undergone oncogenic transformation. We consider it possible that the two proteins, while related to one another in structure, are made on distinct templates within the same cell and derive, at least in part, from distinct sequences in the DNA. Further structural characterization of the two proteins is under way.

Whether *neu* is an allele of the gene that encodes EGF receptor or a second related gene, the similarities between p185 and EGF receptor suggest that the normal *neu* gene product is a growth factor receptor. We speculate that the *neu* transforming protein functions by delivering a mitogenic signal to the interior of the cell, even in the absence of appropriate stimulation by the cognate growth factor—this would be analogous to the finding that the *v-erb-B* gene product lacks the EGF-binding domain of the receptor and must therefore be insensitive to the presence of EGF⁶.

The role of growth factor receptors in spontaneous tumorigenesis was previously inferred from the findings that a human vulval carcinoma cell line (A431)^{13,14}, human squamous cell carcinomas²⁰ and human glial tumours²¹ have large numbers of surface EGF receptors. Taken together with our findings, these results indicate that activation of growth factor receptors may be a frequent occurrence in tumorigenesis. Unlike the *ras* gene product, polypeptide growth factor receptors possess extracellular domains. It is possible that altered growth factor receptors bear novel tumour-specific antigens that can be identified by monospecific antisera. In contrast to most tumour-associated surface antigens, which are probably incidental to the transformation of a cell, the expression of p185 and analogous proteins seems to be intrinsic to the establishment and maintenance of the transformed state. This may make it possible specifically to alter the growth properties of these cells with immunological reagents that recognize determinants expressed on their surface.

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Allelic exclusion and control of endogenous immunoglobulin gene rearrangement in κ transgenic mice

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Hybridomas were produced from spleen cells of κ transgenic mice to investigate expression of the transgenic κ gene, its effect on allelic exclusion and its effect on the control of light-chain gene rearrangement and expression. Our results show that the transgene is expressed normally and that the production of a complete immunoglobulin molecule turns off light-chain gene rearrangement.

B LYMPHOCYTES must correctly juxtapose three heavy-chain gene segments¹ V_H , D_H and $J_H C_H$, and two light-chain gene segments² V_L and $J_L C_L$, in order to produce the two identical heavy polypeptides and two identical light chains that assemble into a complete immunoglobulin molecule. B lymphocytes exhibit allelic and isotypic exclusion—out of two possible heavy-chain alleles and eight possible light-chain alleles (two κ and six λ), a single B cell expresses only one heavy chain together with one light chain³. We seek here to distinguish between two suggested mechanisms for allelic exclusion. A stochastic model assumes that the chance of two functional rearrangements of a heavy- or light-chain gene in the same B cell may be so low that the outcome is exclusion of the other alleles and isotypes in the majority of B cells, because rearrangement is highly error-prone^{4–13}. Another postulated mechanism involves a direct feedback from a functionally rearranged gene or its protein product which shuts off further immunoglobulin gene rearrangements^{14–16}. Details of the possible mechanisms for allelic exclusion have been widely discussed^{4,14–17}.

To determine whether a functionally rearranged light-chain gene influences other immunoglobulin gene rearrangements, we have microinjected a functional κ gene (cloned from the myeloma MOPC-21) into fertilized mouse eggs and produced κ transgenic mice^{18,19}. A functionally rearranged κ gene is therefore present in all B cells of these mice before any endogenous immunoglobulin genes begin to rearrange. In the present work we made hybridomas from spleen cells of these transgenic mice to investigate the expression of the κ -transgene on a single cell level, its effect on allelic exclusion and its effect on the control of light-chain gene rearrangement.

Hybridoma immunoglobulin

Hybridomas were produced from the spleens of three different transgenic mice. We first tested hybridomas for pBR322 DNA (included on the microinjected κ DNA to serve as a marker for the presence of the injected gene) (data not shown) and selected for further analysis 24 clones retaining the microinjected gene and two clones that had lost the chromosome bearing the microinjected gene. Based on secreted κ chains, three types of hybridoma are demonstrated (Fig. 1): cells which secrete the transgenic κ chain, cells which secrete an endogenous κ chain and cells which secrete no κ chain. Of 20 secreting hybridomas, none was found which secretes both transgenic and endogenous κ chains. The transgenic κ secretors (Fig. 1, lanes 17–20) secrete the transgenic κ chain and have only this κ chain in their cytoplasm. The endogenous κ secretors secrete κ chains having isoelectric points different from the MOPC21 κ chain and from each other (lanes 9–16); they can be divided further into two groups on the basis of the presence (lanes 14 and 16) or absence (lanes 10 and 12) of the transgenic κ chain in the cytoplasm. The non-secretors (four hybridomas) secrete no κ chains but have traces of transgenic κ chains in the cytoplasm (lanes 5–8).

Because the samples in Fig. 1 were reduced, we could not eliminate the possibility that the hybridomas are secreting free light chains. The primary structure of immunoglobulin light chains controls their secretion²⁰. Indeed, a single amino acid substitution in the variable region can prevent secretion of free light chain²¹. We have used a property of the MOPC-21 light chain which is not secreted as free light chain but is readily secreted in association with heavy chains²². When the proteins

Table 1 Summary of hybridomas

Type	Hybridoma sample size	Cytoplasmic κ chains		Secreted κ chains		pBR322 DNA	Endogenous κ genes rearranged	Heavy chain mRNA		Heavy chain protein		No. tested for heavy-chain protein	Total
		T	E	T	E			μ	γ	μ	γ		
Transgenic κ_H secretors	1 from mouse 48 3 from 54 7 from 55	+	—	+	—	+	—	4	7	4	6	10/11	11
Endogenous κ_H secretors	1 from 48 1 from 55	—	+	—	+	+	+	2	0	2	0	2/2	2
Endogenous free κ secretors	2 from 48 4 from 54 1 from 55	+	+	—	+	+	+	5	2	—	—	7/7	7
Non-secretors	1 from 54 3 from 55	+	—	—	—	+	2+ 2—	2	2	—	—	4/4	4
Controls	1 from 48 1 from 54	—	—	—	—	—	+	1	1	—	—	2/2	2

Summary of the different kinds and numbers of hybridomas analysed from κ transgenic mice. Cytoplasmic and secreted κ chains were analysed as described in legends to Figs 1, 2; RNA as described in the text. The presence of pBR322 DNA was detected by preparing DNA from hybridomas, dotting onto nitrocellulose and hybridizing with radioactively labelled pBR322 (data not shown).

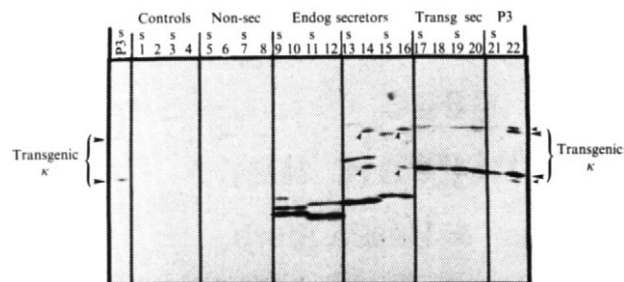


Fig. 1 Isoelectric focusing protein blot of cytoplasmic and secreted κ chains from transgenic hybridomas. Each box of four lanes contains secreted and cytoplasmic κ chains from two hybridomas that represent a particular type. Even-numbered lanes are cytoplasmic proteins; odd-numbered lanes are hybridoma supernatants (S). Lanes P3, 21 and 22 contain the P-3 cell line, a derivative of MOPC-21 that expresses the V_{κ} M.21 light chains. Lanes 1, 2, fusing line SP2/0, producing no κ chain; lanes 3, 4, a control hybridoma that lost the microinjected gene and secretes no κ chains; lanes 5–8, two non-secretors; lanes 9–12, two endogenous κ secretors having no cytoplasmic transgenic κ chains; lanes 13–16, two endogenous κ secretors that have cytoplasmic transgenic κ chains (1) but no secreted transgenic κ chains; lanes 17–20, two transgenic κ secretors.

Methods: Because we needed to use hybridoma technology to isolate a representative B-cell population from the spleens of transgenic mice, three different transgenic mice (numbers 48, 54 and 55; refs 18, 19) were immunized with the polyclonal activators lipopolysaccharide, LPS (5.0 μ g) or sheep red blood cells (0.2 ml of 30% suspension). Mice were given a booster 1 week later, killed 3 days thereafter and spleen cells prepared, fused with the fusing line SP2/0-Ag14 (ref. 29) and hybridomas selected in hypoxanthine-aminopterin-thymidine medium (ref. 34 and S. Settle and J. Brown, personal communication). To analyse cytoplasmic proteins, hybridoma cells were washed twice in phosphate-buffered saline, PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2), pelleted, resuspended in 20 μ l PBS and lysed with 100 μ l of Nonidet P-40 (NP40) lysis buffer (0.5% NP40, 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 50 μ M phenylmethylsulphonyl fluoride). After 30 min on ice, nuclei and debris were pelleted. 15 μ l of hybridoma supernatant and 5 μ l of cytoplasmic protein were reduced with 15 μ l isoelectric focusing (IEF) sample buffer (8 M urea, 1.5 M 2-mercaptoethanol, 0.1 M potassium phosphate, pH 8.0), 0.4% ampholines pH 4–6, 1% Triton X-100, 0.014% ammonium persulphate, 0.1% N, N', N'-tetramethylethylenediamine (TEMED)) and focused to equilibrium on an LKB Multiphor apparatus. After focusing, the proteins were electroblotted onto nitrocellulose in 0.7% acetic acid. Protein blots were blocked with 1% bovine serum albumin, 1% gelatin, probed with a rabbit anti-mouse C_{κ} antiserum, followed by a goat anti-rabbit horseradish peroxidase conjugate and developed with HRP Colour Development Reagent and H_2O_2 according to a procedure from BioRad.

are analysed without reduction of S-S bonds, we find that in both secreted and cytoplasmic proteins of transgenic κ secretors, the transgenic κ chain is associated with a heavy chain (μ or γ) (Fig. 2, lanes 1–4). All hybridomas of this type tested secrete the transgenic κ chain in association with heavy chain and do not secrete free light chains. Endogenous κ secretors that also have cytoplasmic transgenic κ chains do not have associated heavy chains (Fig. 2, lanes 5–8). We conclude that the endogenous κ chains are secreted as free light chains, whereas the transgenic κ chain alone is retained in the cytoplasm. These secretors of free endogenous κ have heavy-chain mRNA (Table 1) but a protein blot of reduced secreted and cytoplasmic protein probed with rabbit anti-mouse μ -, γ - or α -chain failed to detect any heavy chains in any hybridomas of this type (data not shown). Endogenous κ secretors that have no cytoplasmic transgenic κ chains secrete their endogenous κ chains in association with μ heavy chain (Fig. 2, lanes 9–12). Two non-secreting

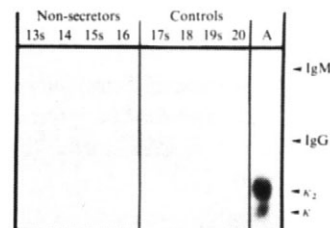
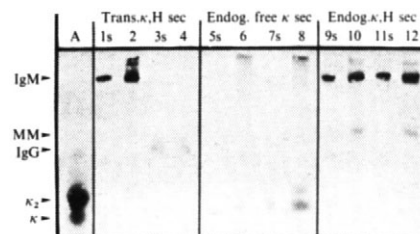


Fig. 2 Protein blot of unreduced hybridoma supernatants and cytoplasmic proteins. Each box of four lanes contains secreted and cytoplasmic proteins from two hybridomas of a particular type. Even-numbered lanes are cytoplasmic proteins; odd-numbered lanes are supernatants. Lane A, P-3 supernatant plus MOPC-41 free light chain (Litton Bionetics). Lanes 1–4, two representative transgenic κ secretors; lanes 5–8, two endogenous free light-chain secretors having cytoplasmic transgenic κ chains; lanes 9–12, two endogenous κ secretors having no cytoplasmic transgenic κ chains; lanes 13–16, two non-secretors (the amount of transgenic κ in the cytoplasm is insufficient to be detected in this gel system, probably because the light chains migrate over a wide range, and are not concentrated into a tight band as in IEF gels); lanes 17, 18, a hybridoma that has lost the transgenic κ gene; lanes 19, 20, fusing line SP2/0. MM, IgM monomer; κ_2 , κ -chain dimer; κ , κ monomer. **Methods:** Supernatants and cytoplasmic proteins were prepared as described in Fig. 1 legend. 20 μ l of supernatant and 15 μ l of cytoplasmic proteins were run unreduced on a 1% agarose, 2.5% acrylamide gel (containing 0.2 M potassium phosphate, pH 7.2, 0.1% SDS, 0.5 M urea, with a running buffer of 0.075 M sodium potassium phosphate, pH 7.2, 0.1% SDS and 0.5 M urea). The proteins were electroblotted in 25 mM Tris base, 192 mM glycine, 20% methanol towards the anode, probed and developed as in Fig. 1. The rabbit anti-mouse κ antiserum does not cross-react with any other heavy or light chains (data not shown).

hybridomas do not produce heavy chains (Fig. 2, lanes 13–16). No κ or heavy-chain protein is observed in a hybridoma that has lost the transgenic gene (Fig. 2, lanes 17, 18) and no κ or heavy chain is detected in the fusing line (Fig. 2, lanes 19, 20).

Hybridoma transcription

In order to determine which κ genes are transcribed, we hybridized total RNA from each hybridoma on Northern blots with cDNA probes for κ , λ and three C_H genes (μ , γ_2b and α) (data not shown). The fusing line SP2/0 contributes C_{κ} transcripts, a V_{κ} M.21 homologous transcript and a λ 1 transcript; transcripts from endogenous light-chain genes therefore cannot be distinguished. However, the fusing line did not produce μ or γ mRNA. All hybridomas produced at least one type of C_H -containing transcript (Table 1). In those hybridomas which produce heavy chains, the heavy-chain transcripts are consistent with the protein detected. Those hybridomas that have no heavy-chain proteins and secrete free endogenous κ chain do have full-sized μ or γ mRNA. Probably, these heavy-chain mRNAs are derived from faulty heavy-chain gene rearrangements and are apparently not translated into recognizable heavy chains.

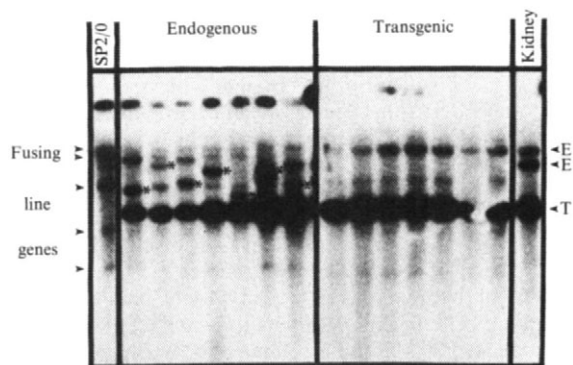


Fig. 3 Southern blot analysis of DNA from transgenic hybridomas. Left, DNA from seven different endogenous κ secretors; right, from seven different transgenic κ secretors. The SP2/0 lane indicates C_{κ} genes contributed by the fusing line, not all of which are maintained in the hybridomas. Kidney DNA from a heterozygous transgenic mouse indicates bands from the *SJL* allele (lower E), *C57BL/6* allele (upper E) and the integrated κ gene (T). The hybridomas shown either were derived from a spleen cell donor homozygous for the *C57BL/6* allele, or have lost the chromosome bearing the *SJL* κ gene. All transgenic DNAs have the 5.2-kilobase *Bam* fragment from the functionally rearranged transgenic κ gene. Note that this fragment is absent from SP2/0. * Endogenous rearranged κ genes. DNA was prepared from hybridoma cell lines as described previously⁵, digested with restriction endonuclease *Bam*HI, electrophoresed at 20 μ g DNA per lane in a 0.9% agarose gel, blotted onto nitrocellulose and probed with C_{κ} sequence (M13-CK)¹⁸.

Characterization of hybridoma light-chain genes

It was important to determine directly whether the microinjected κ gene had an effect on endogenous immunoglobulin gene rearrangement. DNA from all hybridomas was digested with several restriction endonucleases and analysed on Southern blots. We found rearranged endogenous C_{κ} genes among the endogenous κ secretors (Fig. 3). Among the transgenic κ chain secreting hybridomas, we have detected no endogenous κ gene rearrangements on testing with *Bam*HI (Fig. 3), *Eco*RI and *Pvu*II (not shown) restriction enzymes. All DNAs were also restricted with the enzymes *Hind*III and *Sac*I and probed with a DNA sequence corresponding to the 5' half of the J_{κ} - C_{κ} intron (data not shown). Again, no rearrangement of endogenous κ genes was seen in transgenic secretors, whereas hybridomas producing endogenous κ chains did show κ gene rearrangements. No rearrangement of endogenous λ light genes in any hybridomas have been detected using Southern blot analysis of λ light-chain genes with restriction endonucleases *Bam*HI and *Xba*I, and using a V_{λ} probe (data not shown). Finally, Southern blots probed with a J_{H} probe indicate that the different transgenic

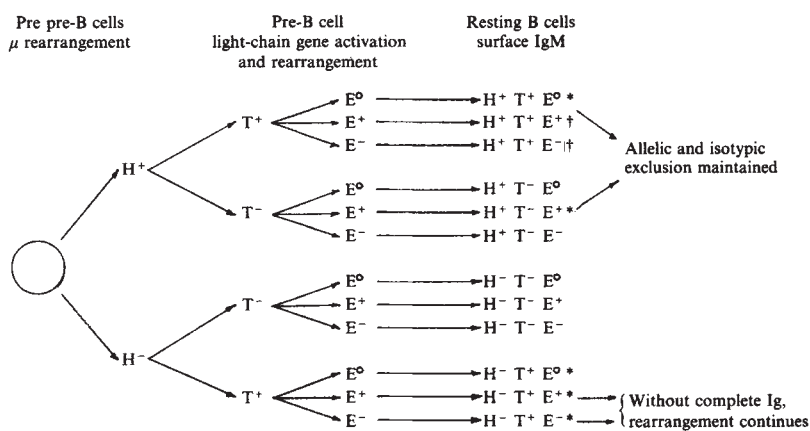
κ secretors have rearranged different VDJ_{H} genes (data not shown).

The analysis of the various types of hybridomas (Table 1) can now be summarized: in transgenic κ secretors, heavy chains are made which then associate with transgenic κ chains and the complete immunoglobulin molecule is secreted; no endogenous light chains are detected and no endogenous light chain genes are rearranged. In endogenous κ secretors that secrete free light chain, no heavy chains are made, the transgenic κ chain is present in the cytoplasm but not secreted and endogenous κ genes are rearranged. In endogenous κ secretors that secrete whole immunoglobulin, heavy-chain proteins are made, the transgenic gene is present but not expressed into protein and endogenous κ genes are rearranged, expressed and assembled into a complete immunoglobulin molecule. In non-secreting hybridomas, heavy chains have not been detected, the transgenic κ is present in the cytoplasm but not secreted, no endogenous κ chains are found in the cytoplasm but endogenous κ genes are present in either a germ-line or rearranged context. These data are consistent with the hypothesis¹⁴⁻¹⁶ that a complete, functional immunoglobulin (not just a functional light chain) has a role in turning off rearrangement. Cell types that have not been detected are first, transgenic κ secretors with cytoplasmic endogenous κ chains, suggesting that in cells where the microinjected gene is completely functional, endogenous κ chains are not produced; second, hybridomas that secrete both transgenic and endogenous κ chains, that is, allelic exclusion is observed; and third, hybridomas that secrete λ light chains. It is known that κ transgenic mice have normal amounts of λ mRNA in their spleen and λ chains in their serum (S. Ogden, K.A.R. and U.S., unpublished observations), but a λ -secreting hybridoma has not been found, perhaps because too few hybridomas have been characterized.

Conclusions

The isolation of B-cell hybridomas that express the microinjected κ gene described here confirms previous findings that the gene is expressed in B cells^{18,19}. We assume that the hybridomas represent a random sampling of B cells present in the transgenic mice; particularly that the transgenic κ secretors represent mature B cells and not pre-B cells immortalized by cell fusion before they had rearranged endogenous κ genes. First, preliminary data from Abelson virus-transformed, transgenic bone marrow cells demonstrate that pre-B cells exist which have μ mRNA but no transgenic κ transcripts (O. Witte, K. Dennis, J. St Claire, R.L.B. and U.S., unpublished results). Second, endogenous κ genes rearrange in hybridomas having no heavy chain but two different κ chains, whereas transgenic κ secretors have neither endogenous κ gene rearrangements nor cytoplasmic endogenous κ chains. Third, several of the transgenic κ secretors produce γ mRNA and protein, which indicates

Fig. 4 Various types of transgenic hybridomas within the context of normal B-cell development. Functionality is defined by the ability to be part of a complete immunoglobulin molecule. H, Heavy chain; T, transgenic κ chain. The $\bar{\cdot}$ symbol does not mean that the transgenic κ genes rearrange, but represents the chance occurrence that a particular B cell inactivates the transgenic κ gene by some event. E, Endogenous light-chain genes. During the normal programme of B-cell development, a transgenic B cell undergoes three major events: rearrange a heavy-chain gene; activate or, by chance, inactivate the transgenic gene; and finally, rearrange (or not rearrange) endogenous light-chain genes. Options at each of these events produce the various types of B cells on the right. Allelic and isotypic exclusion is the result of forming a complete antibody molecule. $\circ$, Unrearranged; $+$, functionally rearranged; $\bar{\cdot}$, non-functionally rearranged gene (nomenclature of ref. 24). *, Cell types found; $\dagger$, cell types not found, that is, rearrangement stops.



mature B cells. Finally, the normal distribution of mature B and T cells in transgenic spleens¹⁹ and the normal histology of spleen and lymph nodes (K. Gollahon and U.S., unpublished observations) reveals no gross abnormalities in lymphocyte development.

We do not know exactly when the transgenic κ gene becomes transcriptionally active relative to normal immunoglobulin gene expression (Fig. 4). The findings with transgenic Abelson pre-B-cell lines (see above) and with normal pre-B-cell hybridomas²³ suggest that μ rearrangement and transcription precedes κ transcription in immature B cells. In mature B cells, unrearranged light-chain genes are known to be transcribed^{23,24}, undermethylated²⁵ and DNase-I sensitive²⁶. If the transgenic κ chain is to have an effect, κ transcription must precede rearrangement. Transgenic κ gene transcriptional activation is shown to occur when normal germ-line κ genes are presumably activated—after μ rearrangement and before endogenous κ gene rearrangement in the scheme in Fig. 4. The isolation of $H^+T^+E^0$ cells (H, heavy chain gene; T, transgenic κ chain gene; E, endogenous light chain gene) but the lack of $H^+T^+E^+$ and $H^+T^+E^-$ cell types indicates that when a functional heavy chain is provided, the transgenic κ gene prevents endogenous light-chain gene rearrangement. The presence of $H^+T^+E^+$ type cells shows that when the transgenic gene is inactivated by chance, endogenous genes can indeed rearrange. Finally, $H^-T^+E^+$ and $H^-T^+E^-$ cell types show that without a heavy chain, the presence of the functional transgenic κ chain alone is insufficient to prevent endogenous immunoglobulin gene rearrangement. The results strongly support the hypothesis¹⁴⁻¹⁶ that the presence of a complete immunoglobulin molecule turns off rearrangement and thus establishes allelic exclusion of immunoglobulin genes. Our data do not address the regulation of λ or heavy-chain gene rearrangement. It seems likely, however, that λ - and heavy-chain gene rearrangements also stop when a complete immunoglobulin is made.

As the history of the hybridomas, or of the B-cells from which they are derived, is unknown, it is possible that the $H^-T^+E^+$ (E^-) cells were derived from H^+ cells. In that case a cell which originally was $H^+T^+E^0$ would have reinitiated rearrangement of endogenous κ genes after the loss of the H^+ chain. This cannot at present be ruled out. It seems likely, however, that the rearrangement machinery becomes permanently inactivated by differentiation into a B cell after the appearance of a complete immunoglobulin molecule. Defining the molecular basis of the feedback inhibition of immunoglobulin gene rearrangement will probably answer this question. If indeed the shutoff of rearrangement is permanent (and if T^+ is a functional protein) then the existence of $H^-T^+E^+(E^-)$ cells suggests that they have never been H^+ and consequently that the presence of heavy-chain protein may not be a prerequisite for light-chain gene rearrangement.

In considering the kind of heavy- and light-chain pairs which have the potential to stop further immunoglobulin gene rearrangement, a distinction must be made between preferential and random association. Clearly, specific light- and heavy-chain combinations are seen in immune responses to antigens like phosphorylcholine. This kind of preferential association, however, is a function of the ability of a pair of heavy and light chains to bind antigen, not of the intrinsic ability of the heavy and light chains to assemble. Several lines of evidence support random pairing of heavy and light chains^{22,27-29}. Preferential association of homologous heavy and light chains has been demonstrated only in competitive situations³⁰⁻³³. Discovery of two full-size κ chains in the myeloma S107 and the association of only one of those with heavy chain indicates that preferential association may have a role in allelic exclusion^{15,16}. In S107, however, the non-secreted κ chain has a deletion of two amino acids including residue 96 (refs 15, 16), which has been found to be critical for association with heavy chains²⁸. As postulated^{15,16}, myeloma S107 does not exemplify preferential association, but shows that a κ rearrangement and polypeptide can be nonfunctional in its inability to associate with heavy chain. Apparently, any heavy-light-chain association can shut off immunoglobulin gene rearrangement, as all heavy chains of transgenic secretors are different (data not shown) and we have found no case of a $H^+T^+E^+$ hybridoma.

Although no myelomas have been described which produce more than one functional κ chain, theoretically such cells might exist. First, it is conceivable that two κ alleles may functionally rearrange and become transcribed at exactly the same time. Second, it is also possible that a particular κ gene, although rearranged, could be transcribed at too low a rate to produce enough κ protein to achieve a threshold level of the heavy-light chain complex required for feedback.

Analysis of these hybridomas provides the first direct evidence for the hypothesis¹⁴⁻¹⁶ that a complete immunoglobulin molecule turns off the process of rearrangement. The mechanism for this shutoff is now being investigated. Our results support the idea that any light chain can associate with any heavy chain, and confirm that the significant mechanism for allelic exclusion is the feedback-mediated shutdown of rearrangement.

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Sequence and expression of transcripts of the human T-cell receptor β -chain genes

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T lymphocytes can produce mRNAs from the first or second constant region of the T-cell antigen receptor β -chain. A 1.3-kilobase (kb) transcript encoding the mature protein contains V, J and one of the two C region sequences, but does not always contain D sequences. A 1.0-kb mRNA, found mainly in immature T cells, does not contain V sequences and can result from transcription that initiates upstream from an unrearranged J element.

B AND T lymphocytes identify and eliminate a large variety of foreign antigens. Understanding the mechanisms of antigen recognition and subsequent activation of these cells is central to our investigation of the immune response. One clear difference between antigen recognition by B and T lymphocytes is that the former can bind antigens by themselves, whereas T cells recognize foreign antigens in conjunction with the major histocompatibility complex (MHC) gene products, a phenomenon known as MHC restriction^{1,2}. Although the immunoglobulin proteins and genes involved in the recognition of antigens by B cells are well characterized^{3,4}, the T-cell antigen receptor, responsible for the binding of antigens by T cells, has only lately been amenable to study.

Human⁵ and murine⁶ T-cell-specific cDNA clones that specify the β -chain⁷ of the T-cell antigen receptor have been isolated. The genes are similar in organization to the immunoglobulin genes with separate variable (V), diversity (D), joining (J) and constant (C) region segments⁸⁻¹¹, rearranging in T cells^{12,13} to produce immunoglobulin-like proteins^{5,6}. There are two closely linked groups of J and C regions in the murine germ-line genome, arranged 5' $J_{\beta 1}-C_{\beta 1}-J_{\beta 2}-C_{\beta 2}$ 3' (refs 10, 11). A similar organization is found in the human genome (B. Toyonaga, personal communication). Each J region is a cluster of independent J segments⁸⁻¹¹ with a closely associated D element on the 5' side¹⁴⁻¹⁶. The distance from the germ-line V genes to the J-C region is unknown¹⁷. We seek here to characterize the transcription products of the rearranged genes of T-cell lines and populations of T cells.

Two constant regions in human β -chain

Analysis of β -chain protein and DNA rearrangement data suggest that there is variability between different T-cell receptor β -chains. To characterize the different transcripts produced from the β -chain region, we constructed cDNA libraries from polyadenylated RNA extracted from the T-cell lines Jurkat or HPB-ALL. Jurkat was of particular interest because of the existence of monoclonal antibodies which recognize both its α - and β -chains (A. Weiss and J. Stobo, personal communication), and because genomic Southern blots show the same pattern of rearrangement as the T-cell line MOLT-3 (ref. 12), from which the original β -chain cDNA clone YT35 was derived⁵. Twelve cDNA clones that hybridized to YT35 were isolated from the Jurkat library and their restriction enzyme sites mapped. These clones fall into two groups: seven that are indistinguishable from YT35, such as JUR- $\beta 1$, and two which are slightly different (Fig. 1). When compared with JUR- $\beta 1$ and YT35, JUR- $\beta 2$ has an extra BglIII site and lacks an EcoRV site. In addition, it is only 0.85 kilobases (kb) long, compared with the full-length size of 1.3 kb for JUR- $\beta 1$.

Nucleotide sequencing of JUR- $\beta 1$ and JUR- $\beta 2$ clones demonstrates that JUR- $\beta 1$ is identical to YT35 (data not shown). This surprising finding indicates that these two different T-cell

lines use the same V, D, J and C region sequences, and explains why Southern analysis shows the same pattern of rearrangement. The sequences of JUR- $\beta 1$ and JUR- $\beta 2$, together with the protein sequence deduced from their only long open reading frame, are compared directly in Fig. 2. JUR- $\beta 2$ extends only a few nucleotides 5' of J. There is some homology between the J segments of the two clones and almost perfect homology in the C protein-coding region. The JUR- $\beta 2$ J segment has a very similar nucleotide sequence to the murine $J_{\beta 2.3}$ element (Fig. 2). In the C region, there are 17 nucleotide differences, only six of which lead to amino acid substitutions. Perhaps the most significant is the transversion at position 992 in JUR- $\beta 1$ that converts the termination codon TGA to a serine codon AGA, producing a protein two amino acids longer. Although the differences between these two C region sequences may reflect polymorphisms between two human chromosomes, it is more likely that they represent transcripts from different pre-existing constant regions in the genome because the 3'-untranslated sequences show no similarities. These two Jurkat transcripts probably correspond to the two distinct but highly homologous C-region segments about 7 kb apart in the human germ-line DNA (B. Toyonaga *et al.*, in preparation). JUR- $\beta 1$ and YT35 are transcribed from the 5' $C_{\beta 1}$ whereas JUR- $\beta 2$ is transcribed $C_{\beta 2}$.

Polyadenylation in JUR- $\beta 2$ occurs at a guanosine residue 20 bases from the polyadenylation signal (marked in Fig. 2). Polyadenylation of the murine β -chain mRNA also occurs at guanosine residues¹¹.

Optional D segments

The T-cell line, HPB-ALL¹⁸, shows a different pattern of rearrangement from MOLT-3 and Jurkat (unpublished data). Comparison of the sequence of a YT35 hybridizing clone, HPB- $\beta 2$, from the HPB-ALL cDNA library¹⁹ with that of JUR- $\beta 2$ indicates that the former is transcribed from the $C_{\beta 2}$ gene, but there are two silent mutations in the C region and a frameshift in the 3'-untranslated sequences (these positions are marked in Fig. 3). We suggest that these changes result from polymorphisms in the human population. A comparison of the nucleotide and deduced protein sequences of HPB- $\beta 2$ with JUR- $\beta 1$ is shown in Fig. 3. The homology between the C coding regions is very high, but there is only random homology in the untranslated region, as is the case between the $C_{\beta 1}$ and $C_{\beta 2}$ regions of JUR- $\beta 1$ and JUR- $\beta 2$. The homology in the J segment (45% overall) is strongest in the middle, which is the most highly conserved region in the murine J segments at both the nucleotide and amino acid level. The J region has 74% identity (35/47) with the murine $J_{\beta 2.5}$ element¹⁰. The V regions are different, but clearly related. The regions of highest homology are clustered around the 3' end, where there is a cysteine residue in the protein sequence, and around position 305 in JUR- $\beta 1$. To align the V regions properly, a gap of 15 nucleotides (five codons) must be introduced into HPB- $\beta 2$ between the J and V sequences; this

histocompatibility locus antigen (HLA-A/B) typing (J. Falk, unpublished observations) and OKT surface marker typing²³; nor is it possible that the original cDNA clones were interchanged because we cloned YT35 into the *Bgl*III site⁵ of pFP502EB5 (ref. 24) whereas JUR- β 1 was cloned into the *Pst*I site of pBR322. The strong hybridization of the YT35 V-region to Jurkat RNA (Fig. 4b) provides independent evidence that YT35 and Jurkat use the same V_{β} -gene fragment. We do not know whether the production of this same mRNA in two transformed lines is related to tumorigenesis.

Preliminary examination of the β -chain rearrangements in bone marrow samples of 13 T-cell acute lymphoblastic leukaemia patients suggests a limited number of rearrangement patterns²⁵. This does not represent a limited repertoire of possible recombinations. There may be some preferred rearrangements in early thymocytes after they have become transformed, or some rearrangements may predispose the cells to transformation. This latter possibly is of particular interest in view of the hypothesis of AKR retroviral leukaemogenesis of Weissman and co-workers²⁶ which suggests that autostimulation of the T-cell receptor leads to a proliferative state during which the cell is more susceptible to transformation.

It has been shown that 1.0-kb mRNAs from thymocytes contain sequences from 5' of a D_{β} element rearranged to a J_{β} segment but not a V_{β} gene^{14,15}. In contrast, this transcript from the cell line Jurkat, represented by cDNA clone JUR- β 2, appears to contain sequences 5' to a J_{β} element rearranged neither to a D_{β} element nor to a V_{β} element. Unfortunately this clone does not extend further 5', but the limited sequence available is identical to the heptamer sequence 5' of the murine $J_{\beta 2.3}$, but different from $D_{\beta 1.1}$ (refs 14–16), $D_{\beta \text{YT}35}$ and the highly conserved 3' end of $V_{\beta \text{M}3.2}$ and $V_{\beta \text{H} \text{P} \text{B}1}$.

The chromosomal origin of this mRNA is unknown. The mechanism activating transcription of the functionally rearranged β -chain may induce also an aberrant transcript from the β -chain locus on the other chromosome which may have

D - J rearrangement; the activation may, however, be localized to one of the two chromosomes, including both $C_{\beta 1}$ and $C_{\beta 2}$. The question of whether the β -chain locus on both chromosomes can be simultaneously transcribed can be resolved with a cell line that uses $C_{\beta 1}$ for the 1.0-kb mRNA and $C_{\beta 2}$ for the 1.3-kb mRNA, where $C_{\beta 1}$ would be deleted.

Two major concerns of immunologists are the mechanisms of elimination of self-reactive T cells during T-cell ontogeny and of MHC restriction. Could the 1.0-kb mRNA be involved in these processes? The discrete and constant size of this transcript in thymocytes, Jurkat, MOLT-3 and other cells implies that there is a specific point for transcription initiation, unique to a particular cell type. Apparently, transcription initiation may occur upstream from an unrearranged J segment or from a J element recombined with a D segment. We do not know whether the smaller message is expressed simultaneously with the larger one in thymocytes as it is in MOLT-3 and Jurkat, which are transformed and thus may be abnormal, or whether pack cells have only one species of 1.0-kb mRNA. The T-cell-specific message clones by Tonegawa's group²⁷ also has a smaller size class. This may be a common property of this gene family.

An interesting difference between thymocytes and mature peripheral T cells is the preferential expression of $C_{\beta 2}$ in the thymocytes, in contrast to the situation in BALB/c mice, where $C_{\beta 1}$ is the most prevalent thymic transcript¹¹. We do not know whether this is due to species differences, or variability between individuals. Further characterization of β -chain expression should assist us in our attempts to understand the mechanisms by which a functional T-cell receptor protein is generated.

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LETTERS TO NATURE

Inflation in the universe and time asymmetry

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I previously argued that the origin of time asymmetry is to be sought in the processes that characterized the very early cosmological epochs, and that the recent inflationary scenario provides a crucial element in our understanding of this origin¹. Here, I develop this thesis further, in the light of criticisms by Page². I argue in particular that any of the “initial states” that appear to be permitted by quantum gravity, if succeeded by a period of inflation, would have wound up the universe to the state from which, as indicated by increasing entropy, it is now winding down.

The universe is ordered in two distinct ways. First, matter (for example stars) is generally found in a state rather far from thermodynamic equilibrium. Second, the universe is mysteriously regular on a larger scale, with only a moderate degree of structure, occasioned by the clumping of material into stars, galaxies, galactic clusters and so on. The cosmological gravitational field is therefore rather smooth. Both these forms of order are gradually degenerating, as matter is processed and loses free energy (as in stellar evolution for example) and as gravity causes the irreversible collapse of objects and systems towards, ultimately, a black hole state. This universal tendency may be considered as a one-way approach to thermodynamic equilibrium and maximum entropy in accordance with the second law of thermodynamics³. But the irreversible rise in cosmic entropy associated with this decay raises the question of how the universe achieved this order initially.

The central problem in addressing the above question is not in doubt. If the fundamental laws of physics are time-asym-

metric, then the observed time asymmetry of the world can only be explained by appealing to a special choice of initial state. (I ignore here the possibility of T-violating laws, as they are irrelevant to the issues under discussion.) Two questions then arise. First, can we identify the physical mechanisms whereby the special initial state evolves into the present (and projected future) state of the universe in a time-asymmetric fashion? And second, can we find reasons why the special initial state should be regarded as unremarkable, 'natural' or even inevitable? The first of these latter two questions is physical, the second essentially philosophical, and strongly model-dependent.

The first person to address questions 1 and 2 was Boltzmann, who treated a simple model gas. He was able to prove, in his famous H-theorem⁴ that, under the impact of intermolecular collisions, the entropy of the gas would rise with time whenever it found itself in a condition of 'molecular chaos'—that is, lacking correlations in the positions and motions of the molecules³. Applying an extrapolation of this theorem to the universe as a whole demands an answer to the second question. One might suppose that if the universe were initially in a state of 'cosmic molecular chaos', then subsequently the entropy would rise, during which phase the universe would behave time-asymmetrically. But now we come up against the question of how the universe achieved its state of 'molecular chaos' at less-than-maximum entropy in the first place.

One unscientific and metaphysical answer is simply to assume that the universe was made that way, that is, created in a special initial state. Another answer (the one suggested by Boltzmann) is that, in a universe which is infinite in time and/or space, and subject to time-symmetric laws, such a state is bound to occur purely by chance fluctuations (and occur infinitely often). The frequency of such a near-miracle is absurdly small, but—to use an anthropic explanation—because the existence of life depends on a source of free energy, we could not possibly find ourselves living in the much-more-probable state of thermodynamic equilibrium.

The above reasoning unfortunately contains a serious flaw. There seems to be no obvious reason why a fluctuation region should be of Hubble proportions in order that observers arise. Because the magnitude of fluctuations is exponentially related to their (im)probability, it would seem to be overwhelmingly more likely that a random observer would find himself in, say, a one-galaxy, or even a one-star, universe than in one populated by myriads of similar systems⁵. In short, the anthropic principle seems to suggest that we should observe a universe of minimal order consistent with the existence of observers.

It is against this general background of ideas that the inflationary universe scenario must be assessed. Briefly the scenario goes like this^{6,7}. At some early moment the universe finds itself in a quantum state known as a false vacuum, which is characterized by a huge energy density and negative pressure. The gravitational effect is identical to that of Einstein's cosmological constant, so that the universe embarks on a phase of exponential expansion (inflation). During this time the universe resembles a portion of de Sitter space. Eventually the inflationary phase ends, and the energy from the false vacuum is deposited into matter and radiation fields. At this stage, irregularities present initially will have been smoothed to insignificance by the enormous distension of space. Subsequently the universe evolves according to the standard hot big bang model.

In ref. 1 I argued that the process of inflation is responsible for 'winding up' the cosmic mechanism, by ensuring that the post-inflationary state is relatively smooth, hence enjoying a gravitational entropy much less than the maximum permissible. The apparent paradox that the universe has thereby progressed from a high to a low entropy state during the inflationary phase is resolved by noting that the universe is not a closed system (even if spatially finite) because free energy is not conserved in cosmology. On account of the negative pressure of the false vacuum, non-gravitational energy is actually created (per unit comoving volume) during inflation.

Boltzmann was faced with the question of how the universe achieved a state of (1) less than maximum entropy and (2) no correlations (molecular chaos). The situation here is directly analogous. We too have to explain how the universe arrived in the false vacuum state initially, and why that state had no (or at least few) correlations.

Page has emphasized the fact that de Sitter space is time-symmetric². As the inflationary phase is finite in duration, the patch of de Sitter space corresponding to this phase will have an 'in' and an 'out' region. According to the inflationary scenario, metric perturbations (irregularities) about the de Sitter background present in the in region will decay during the inflationary phase, and so will be infinitesimal in the out region. However, because of the time symmetry, for every solution of the gravitational field equations which corresponds to decaying perturbations, there will be a time-reversed solution which describes growing perturbations. (The proof of Boucher and Gibbons⁸ that the metric approaches de Sitter space exponentially fast assumes an infinite future with non-zero cosmological constant, thus breaking the time symmetry.) A sufficient, though not necessary, condition for decaying perturbations is the absence of correlations in the metric perturbations in the in region. (Roughly speaking, the perturbations will only grow if they conspire to organize themselves over a large spatial region in a cooperative fashion.) The situation is directly analogous to a Boltzmann-type model gas in a state of molecular chaos.

It is now necessary to explain why it is reasonable for the universe to have been in a state with no correlations initially. My argument is an adaptation of that frequently used³ to explain why randomly selected branch systems in low entropy states are exceedingly likely to be in a state close to molecular chaos.

To repeat the molecular argument first: imagine a gas enclosed for all time in an impenetrable box. The gas spends the overwhelming proportion of its time very close to thermodynamic equilibrium at maximum entropy $S_{\max}$, but occasionally makes small excursions from equilibrium on account of random fluctuations. The theory of fluctuations demands that small fluctuations, whilst rare, are nevertheless exceedingly more probable than large fluctuations.

Suppose now that the box of gas were to be equipped with a sensor that flashed a light whenever the gas achieve a state having a particular pre-selected value of the entropy, say S , where $S < S_{\max}$. It then follows that, on inspection, this state would almost certainly be very close to a local minimum in the entropy curve, close to the point of maximum excursion from $S_{\max}$. The reason for this is that there are vastly more fluctuations which just reach S from $S_{\max}$ than there are larger fluctuations that pass through S on their way to still lower values of the entropy. Hence a randomly selected low-entropy state is overwhelmingly likely to be at a local entropy minimum.

Now it may be shown that³ the condition of molecular chaos holds at the minimum point of an entropy fluctuation (which is, of course, a point of time symmetry with the entropy increasing in both forward and reverse time directions). It follows that a randomly selected low-entropy state will almost certainly be one essentially devoid of molecular correlations. This argument explains why it is that in a realistic physical situation, where a box of gas does not remain undisturbed for all time but instead is formed as a quasi-isolated branch system⁹ separated off from the wider environment, the entropy of the gas is always observed to rise. It is because, if the branch system is formed at random (and not at one of those rare instances where the molecules are conspiring to produce a large fluctuation to still lower entropy), it will almost certainly be in a state of no correlations (molecular chaos), for which the conditions of Boltzmann's *H*-theorem apply.

The case of de Sitter space is very closely analogous to the box of gas. de Sitter space may be considered to be a state of gravitational thermodynamic equilibrium, and indeed there is even an associated temperature¹⁰. (It may seem puzzling that the highly-symmetric de Sitter space is described as a maximum entropy state, whereas earlier it was stated that self-gravitating

systems tend to grow irregularities as they evolve towards thermodynamic equilibrium. The difference arises from the crucial role played by the cosmological constant. When such a constant is present the tendency for a self-gravitating system to grow clumpy is reversed; the exact opposite situation obtains, as explained in ref. 1.) Quantum effects will cause fluctuations to occur about the strict de Sitter background. Again, large fluctuations are much rarer than small fluctuations. At the minimum point of such a fluctuation the perturbations will be uncorrelated, corresponding to 'cosmic molecular chaos'. By the same reasoning as above, a randomly chosen perturbed state will almost certainly be such a state of no correlations at the minimum of a fluctuation curve. This state is then one in which the perturbations will decay rather than grow whichever direction of time is chosen as forward. Of course, in the inflationary scenario, a past-forward time convention is imposed by the very different nature of the in and out regions, the out region corresponding to a hot Friedman universe and the in region of a form to be discussed below.

Although the state of molecular chaos in a gas is sufficient for it to be shown, via the H -theorem, that entropy subsequently increases, such a state is not necessary. Indeed, it is easily demonstrated³ that if a gas is in a state of molecular chaos at some instant, it will not be in that condition at subsequent instants. Thus the H -theorem cannot be used to prove the continuous approach of a molecular system to equilibrium. Nevertheless we all know that the gas will so equilibrate, in spite of the correlations introduced during the climb towards maximum entropy as a result of the very molecular collisions that drive it there. Similarly, although a zero-correlation state is sufficient for the decay of perturbations in de Sitter space it is not necessary. Many states with some correlations will also tend towards de Sitter space. Thus there is a very much larger class of possible initial states consistent with the standard inflationary scenario.

To complete the inflationary picture it is necessary to have some idea of how the universe achieved its initial state, prior to the inflationary phase. At this point one must distinguish between two versions of the inflationary theory. In the first version the universe cools into the false vacuum from a pre-existing hot Friedman-like phase. While, as has been argued above, it seems likely that if such universe embarks upon inflation then any initial irregularities will decay, inflation will only proceed in any case if there is already a reasonable degree of regularity; inhomogeneity and anisotropy tend to suppress the inflationary mechanism¹¹. In spite of this, there will exist regions of space where by chance fluctuations the conditions are right for inflation to proceed. Thus, amid a sea of chaos, here and there bubbles of space will inflate. It only then remains for us to explain why we happen to be living in such a region. But it is only in the post-inflationary bubbles that large-scale order appears, itself an essential prerequisite for life and observers. We could not possibly exist amid the chaos outside of our quiescent 'oasis'. This is, of course, essentially a resurrection of Boltzmann's explanation of time asymmetry, but in a modern guise which circumvents the original objections for the following reasons.

First, the effect of inflation is to enormously amplify the initial chance fluctuation to moderate smoothness. The inflationary mechanism permits a fairly modest fluctuation to trigger the spontaneous growth of a huge ordered region containing a vast quantity of negative entropy relative to the locally maximum attainable entropy. Thus, inflation acts as a negative entropy amplifier. (The precise way in which this 'entropy gap' is widened by inflation has to do with the non-conservation of useful energy, and was explained in ref. 1.) This is in contrast to Boltzmann's original concept, in which the very large degree of negative entropy observed in our region of the universe today had to be generated *in toto* by a far less probable conjunction of fluctuations.

Second, in the context of the inflationary model, it appears that we do live in a minimum fluctuation region. It is a feature

of the inflationary theory that you either get a whole bubble universe or nothing. That is to say, once a modest fluctuation has triggered off inflation in a region, the dynamics of the inflationary mechanism will inevitably produce a bubble universe of extra-Hubble proportions. (The size of the fluctuation region is determined by the parameters of the theory, which fix such things as the 'roll-over' time¹².) Thus, a universe of the scale we observe is either produced in one blow, or not at all. So there is no mystery as to why a Hubble-scale fluctuation region represents a vast over-provision of negative entropy as far as life on Earth is concerned.

Finally, I turn to the other version of the inflationary scenario wherein the universe arrives at the threshold of inflation as the result of a quantum tunnelling process 'from nothing'. Vilenkin has proposed¹³ that the universe was 'initially' in a quantum state in which there is no classical spacetime, a state he refers to as 'nothing'. As a result of quantum fluctuations the universe then 'tunnels' into a de Sitter space from which inflation may proceed as usual. The tunnelling amplitude is discussed using a Euclideanized path integral for a quantum description of the gravitational field. Using semiclassical techniques borrowed from quantum mechanics and quantum field theory, Vilenkin argues that the most probable spacetime to emerge 'from the tunnel' is homogeneous and isotropic. Furthermore, the associated scalar field, which subsequently drives the inflationary mechanism, is found to be in the required false vacuum state. Although Vilenkin's original discussion was speculative and largely heuristic, his results have received support from the more detailed work of Moss and Wright¹⁴ on the wave function of the universe.

Similar ideas to those of Vilenkin have been proposed independently by Hawking¹⁵. The key element here is the suggestion that the quantum state of the universe might be one that is uniquely defined by a 'natural' mathematical prescription. This prescription has been discussed by Hartle and Hawking¹⁶, and in essence selects something akin to a minimum energy state of the universe. Hawking and Luttrell¹⁷ argue that the Hartle-Hawking ansatz requires an isotropic universe. Similar arguments can be used to select a homogeneous initial state (I. G. Moss, personal communication).

Thus, in this version, the universe is automatically driven towards something like de Sitter space by quantum processes, from which inflation proceeds in the usual way. Here it is not necessary to invoke an anthropic argument to select an ordered 'bubble' from the rest of the universe.

To what extent can these (necessarily imprecise) remarks provide a basis for an explanation of time asymmetry in the universe? The word 'explanation' can have two meanings. First it can mean 'tracing the origin'. Certainly the inflationary scenario provides a very plausible description of how the universe became 'wound up' as a result of quantum and gravitational processes. Once thus wound, the time asymmetry of the universe subsequently is assured, as explained in ref. 1.

But we can still ask why the universe started out in the required pre-inflationary state in the first place. This is a deep philosophical issue, for we are obviously in danger of falling into an infinite regress. Conceivably one could show quantum mechanically that the probability of producing a universe already in its maximum entropy state is exceedingly small. Indeed, it seems very plausible that a universe which originates from some sort of quantum gravitational fluctuation would be of Planck dimensions and Planck temperature, rather than Hubble dimensions and full of black holes. In the case of the version of inflation that starts from a Friedmann-like phase, this pre-inflationary state is usually taken to originate in a 'little bang' singularity, and the question of 'why that Friedmann-like state' reduces to the daunting (and perhaps unphysical) problem of what comes out of a spacetime singularity¹⁸.

In both versions, then, we have the difficulty that the chain of explanation is pushed back so far that it disappears off the edge of spacetime, either into a quantum tunnel or a singularity. Perhaps one has to stop there; after all, we can hardly make

much sense of the question 'why that state in the first place?' when there is no longer any classical spacetime for there to be a 'first place'. In this respect, the Hartle-Hawking approach to defining a unique state for the universe is attractive because it makes no use of the concepts of initial or final. Thus, we are forced to change the nature of the questions from the traditional 'why that spatial state in the first place?' to 'what is the most natural state of spacetime?'

My explanation for time asymmetry from inflation thus depends on which of the two alternative scenarios one considers. In the tunnelling version one adopts some criterion of 'naturalness' to select a very special initial state for the whole universe, in which case inflation merely acts to drastically reduce the improbability of such a state by permitting a wide class of initial states to develop into something like the universe we now see. In the other version, one can accept an arbitrary initial state but appeal to anthropic selection of an atypical region. Here, inflation is a crucial element in the argument because it explains why the fluctuation region necessary for observers to evolve is so large. In this sense, then, I maintain that inflation does explain time asymmetry.

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Two new young galactic supernova remnants

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Substantial reductions¹⁻³ in the distances inferred for the remnants of several 'historical' supernovae, coupled with a galactic supernova rate of 1 every 25 yrs or so⁴, have raised the possibility⁵ that many young but distant galactic supernova remnants may be missing from present catalogues. We have now used the Very Large Array (VLA) and the Cambridge 5-km telescope to observe 32 small-diameter objects selected from galactic plane radio surveys. These observations reveal both a new young shell remnant (which, at only ~1 arc min in extent, is the smallest angular size galactic remnant yet discovered), and a very peculiar radio source, probably a supernova remnant only a few hundred years old, which is still in the free expansion phase.

Supernova remnants mark the violent endpoints of stellar evolution, and studies of them are relevant not only to our understanding of supernovae and pulsars, but also to that of the interstellar medium and the generation in fast shocks of relativistic particles and magnetic fields. These problems can be tackled either statistically or by detailed investigations of

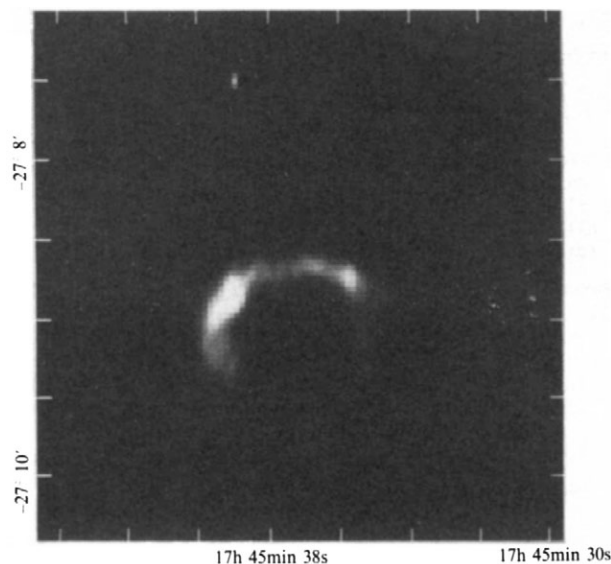


Fig. 1 CLEANed map (500 components) of G1.9+0.3 at 4.9 GHz, restored with a 4.4×2 arc s² beam. The plotted range, black to white, is -1 to 9 mJy per beam.

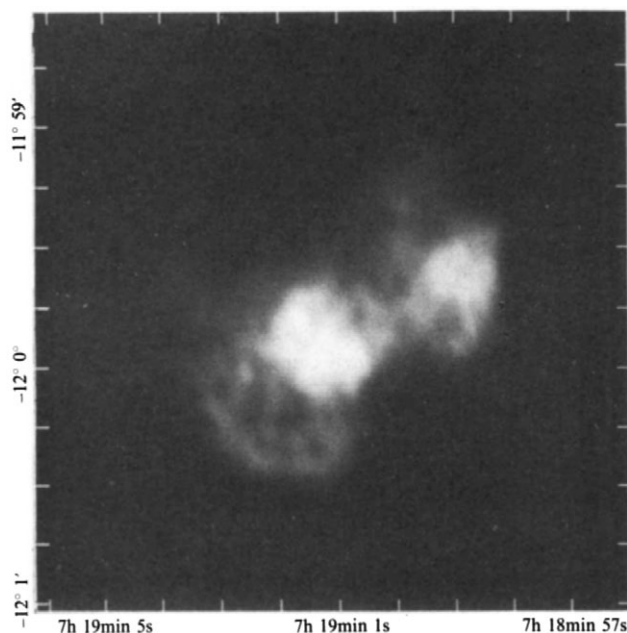


Fig. 2 CLEANed map (2000 components) of G227.1+1.0 at 4.9 GHz, restored with a 6×4 arc s² beam. The plotted range, black to white, is -1 to 9 mJy per beam.

individual remnants, among which the relatively bright remnants of historical supernovae (see ref. 6) are particularly important. Some of these, such as Cassiopeia A and the Crab Nebula, are amongst the brightest radio sources in the sky, so it is unlikely that any similar remnants in the Galaxy could have been missed. Others, such as 3C58 and the remnants of Tycho's and Kepler's supernovae, would have flux densities (at 1 GHz) of only ~1 Jy, and be <1 arc min in extent if they were removed to the other side of the Galaxy (~20 kpc). Present catalogues contain no such remnants, but existing galactic radio surveys, with only moderate resolution (a few arc minutes), do contain many unresolved or barely resolved sources with flux densities and spectral indices appropriate for young but distant supernova remnants. Few of these sources have been observed with higher resolutions.

Clark and Crawford⁷ list 513 small-diameter (<1.5 arc min) sources in the southern galactic plane ($|b| < 3^\circ$, declination below $+17^\circ$) from the Molonglo survey⁸, with flux densities >0.6 Jy at 408 MHz. Sources on this list which are brighter than 1 Jy (and

Table 1 Observed sources

Galactic coordinates		Comments
<i>l</i>	<i>b</i>	
Observed with the 5-km telescope at 2.7 GHz		
49.66	+2.91	Several sources*
51.22	-1.42	Single compact source
53.43	-1.28	Single compact source
193.63	-1.52	Close double
Observed with the VLA, 'C' array, at 5 GHz		
1.87	+0.33	Shell remnant, ~1 arc min in diameter
6.76	+0.92	Close double
9.82	-0.99	Single compact source
11.97	+0.19	Double, ~1 arc min, with bright core
20.71	+2.29	Single compact source
21.05	+1.96	Single compact source
25.26	-0.14	Three sources
24.88	-1.71	Close double
32.26	+1.17	Single compact source
32.95	-1.79	Close double
39.56	-0.04	Single compact source
39.42	-0.59	Close double
197.14	-0.85	Close double
201.07	-1.02	Single compact source
203.52	-1.40	Single compact source
201.53	+0.51	Single compact source
214.70	-1.75	Close double
217.78	-2.95	Single compact source
213.13	+0.57	Double, ~1 arc min
223.54	-2.82	Close double
226.52	-2.92	Single compact source
224.66	-1.72	Single compact source
229.87	-0.64	Single compact source
230.08	-0.62	Single compact source
227.09	+1.02	Strange source, ~1 arc min in extent
238.40	-2.46	Single compact source
237.32	-1.77	Single compact source
245.16	+2.77	Single compact source

* Also observed by Helfand *et al.*¹⁰.

some of the fainter ones) have been observed further⁹ with the Parkes 64-m radio telescope at 5 GHz, so that spectral indices are available (with errors <0.1) for 325 of these small-diameter sources. From these lists we selected candidate young supernova remnants on the criteria that the declination should be above -30° (so that reasonable maps could be made with the VLA), and the spectral index, α (where $S(\nu) \propto \nu^{-\alpha}$), should be between 0.0 and 0.8, which covers the expected range for both filled-centre ($\sim 0.2 \pm 0.2$) and shell-type ($\sim 0.6 \pm 0.2$) remnants. (Three other sources, each with flat spectra ($\alpha < 0.4$), satisfy these criteria, but these have already been observed by Helfand *et al.*¹⁰ in their unsuccessful search for new young 'filled-centre' remnants.)

Table 1 lists the galactic coordinates (from ref. 9) of the 32 sources we have observed. The four sources above declination $+15^\circ$ were observed in April 1984 with the Cambridge 5-km telescope at 2.7 GHz, with a resolution of $\sim 4 \times 14$ arc s (ref. 2), and the other 28 were observed in May 1984 with the VLA in C configuration at 4.9 GHz, with a resolution of ~ 4 arc s. Most of these objects are compact single or close double sources, and are presumably extragalactic. Four of them are, however, extended on a scale of ~ 1 arc min: two are extended double sources and are probably also extragalactic. The other two extended sources are discussed below.

G1.9+0.3 (Fig. 1) is a shell source with a radio surface brightness of $\sim 7 \times 10^{-20} \text{ W m}^{-2} \text{ Hz}^{-1} \text{ sr}^{-1}$ at 1 GHz, which is slightly less than those of Tycho's and Kepler's supernova remnants, but appreciably larger (by a factor of 25) than that of the remnant of SN1006. Its radio spectral index is ~ 0.7 , comparable with that of known young shell remnants. It is thus

almost certainly a supernova remnant. Although this source is nearly circular, the intensity of radio emission varies considerably around the shell and is strikingly similar to Kepler's supernova remnant¹¹ in this respect. A maximum galactocentric radius of ~ 20 kpc gives an upper distance limit of ~ 30 kpc for this object, which then implies a diameter of <10 pc. (Compare this with Tycho, Kepler and SN1006 supernova remnants, with diameters of 5, 4 and 9 pc, respectively.) We conclude that the age of this remnant is between 400 and 1,000 yr.

G227.1+1.0 (Fig. 2) is a very peculiar source, unlike known galactic supernova remnants or extragalactic sources. Its surface brightness is $\sim 2 \times 10^{-19} \text{ W m}^{-2} \text{ Hz}^{-1} \text{ sr}^{-1}$ at 1 GHz, which is similar to that of known young remnants such as those of Tycho and Kepler and 3C58. Its radio spectral index is ~ 0.6 , and the non-thermal nature of the radio emission is confirmed by its high degree of polarization (Fig. 3).

If this is a galactic object, it is likely to be closer than ~ 10 kpc, because its galactic longitude is 227° , towards the galactic anti-centre. Its linear size would then be <4 pc, consistent with a supernova remnant only a few hundred years old.

Our interpretation of this source is that it is the result of a very recent supernova, at most a few hundred years old. The slightly limb-brightened lobes of fainter emission to the east and west would be a ring of ejecta from the supernova explosion which is beginning to be decelerated by its interaction with the surrounding interstellar medium. Furthermore, if the Faraday rotation to the source is small at 5 GHz, then the magnetic field alignment is tangential to the edge of the emission in the eastern lobe, as might be expected. The brighter central plateau of emission is, in this interpretation, a young 'filled-centre' remnant, a plasma bubble powered by a pulsar, the compact remnant of the supernova explosion (which may be related to the one of the compact features visible in the brighter region in Fig. 2).

If both objects are young but distant galactic supernova remnants, their parent supernova may have been observed historically, and they should also be observable as X-ray sources. However, there are no historical records of 'guest stars' (see ref. 6) in the direction of either object, and neither is there a catalogued X-ray source¹².

The search for and detection of new young galactic supernova remnants described above confirms that present catalogues are incomplete with respect to young but distant remnants. Until this is rectified, statistical studies of galactic supernova remnants will be severely limited. On the other hand, the detection of further young supernova remnants will provide more objects that are relatively bright and easily studied.

We thank the staff of both the 5-km telescope and the VLA for their help with the observations. The VLA is operated by Associated Universities Inc., under contract with the NSF.

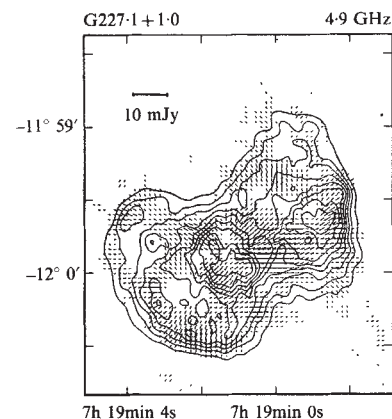


Fig. 3 CLEANed map of G227.1+1.0 restored with a 6×4 arc s² beam. Contours of Stokes parameter *I*, at $0.84 \times (1, 2, \dots, 10)$ mJy per beam with polarization *E*-vectors superposed where the polarized intensity exceeds 0.1 mJy per beam.

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Hyperbolic umbilic diffraction catastrophe and rainbow scattering from spheroidal drops

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The characterization of short-wavelength scattering phenomena has been advanced by the study of diffraction catastrophes^{1,2}. Examples include optical phenomena¹⁻⁵ and molecular collisions⁶. The most familiar example of a diffraction catastrophe is the ordinary rainbow⁷⁻⁹. The angular variation of the scattering from a spherical drop locally corresponds to that of a fold-diffraction catastrophe^{1,2}, a result most clearly seen with monochromatic illumination. We have studied the scattering from drops whose shape closely approximated that of an oblate spheroid with the short axis—the symmetry axis—vertical. The drops were illuminated by a horizontally propagating gaussian beam with a wavelength $\lambda = 633$ nm. These drops were observed to scatter in the horizontal rainbow region with patterns like those of hyperbolic-umbilic (classification D_4^+) diffraction catastrophes^{1,5,6}. Visible D_4^+ diffraction patterns observed previously include light transmitted by frosted glass surfaces¹⁰ and by liquid lenses clinging to tilted glass plates^{1,4}.

Figure 1 shows the ray diagram of the primary rainbow of a spherical drop. For the once-reflected (twice-refracted) rays there is a minimum scattering angle of $\theta_R \approx 138^\circ$ and the associated ray is known as the Descartes ray. When the scattering angle θ is between θ_R and 166° , there are two rays of the class shown. The rays labelled 1 and 2 are for $\theta \approx 152^\circ$. In the two-ray region, intensity oscillations produce the supernumerary arcs⁷⁻⁹. Figure 1 is also applicable to rays which lie in the horizontal equatorial plane P of the spheroidal drops; P cuts the drop's surface in a circle and both the incident ray and the local surface normals lie in P.

In our apparatus (see Fig. 2), a horizontal metal plate was coupled to a piezoelectric vibrator which was driven at a frequency of 27 kHz. A curved reflector (not shown in Fig. 2) was placed above the plate's centre so as to establish an acoustic standing wave in the air below the reflector. Drops of distilled water could be levitated in this wave by an upward-directed acoustic radiation pressure. (The levitator is similar, in principle, to one described in ref. 11.) Figure 3 shows a levitated drop. The oblate shape is due to the spatial distribution of the radiation pressure¹². In addition to the deformation evident in Fig. 3, the drop vibrates at 27 kHz with surface displacements which are too small to affect the light scattering. The drop's diameter will be denoted by D in plane P, and by H along the

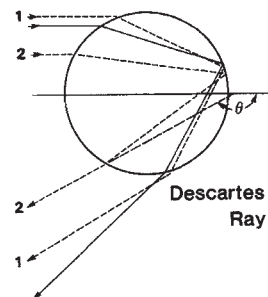


Fig. 1 Rays through either a spherical drop or through a spheroidal drop in the equatorial plane.

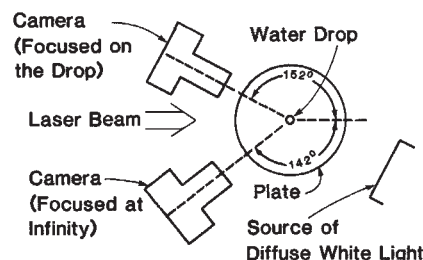


Fig. 2 Schematic view of the apparatus from above. The drop is held 11 mm above the plate and the optical axes of the cameras lie near a horizontal plane containing the drop. The diffuse light is usually turned off.

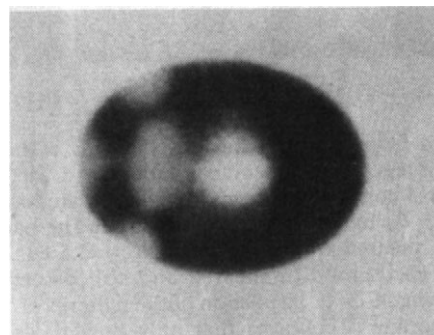


Fig. 3 Photographs of an acoustically levitated drop having a diameter $D = 2.3$ mm. The drop was illuminated both with diffuse light and the laser beam. The four patches on the left are due to refracted laser light.

vertical symmetry axis. Raising the ultrasonic amplitude increases D/H .

The scattering was photographed for randomly polarized illumination from a He-Ne laser. The camera was focused on infinity so that the pattern recorded was equivalent to that in the far field (which is at distances $r \gg D^2/\lambda$ from the drop). Horizontal and vertical coordinates in each photograph were linear in the horizontal and vertical scattering angles θ and β , where β is relative to plane P. The camera viewed the region: $136^\circ < \theta < 148^\circ$, $-6^\circ < \beta < 6^\circ$.

Figure 4a shows the scattering from a slightly nonspherical drop having $D = 1.9$ mm. The intensity modulations are similar to other photographs of supernumerary arcs with laser illumination¹³. Within experimental uncertainties, their spacing is as predicted by Airy theory^{7,9}. Figure 4b-f are the patterns for another less nearly spherical drop. For Fig. 4b the drop had $D = 1.39$ mm and $D/H \approx 1.23$; smaller values of D/H gave patterns similar to Fig. 4a. Changes in the pattern with changes in D/H were also viewed through the camera's lens by eye.

Figure 4d manifests the parallelogram and caustic structures characteristic of focal section patterns of D_4^+ diffraction

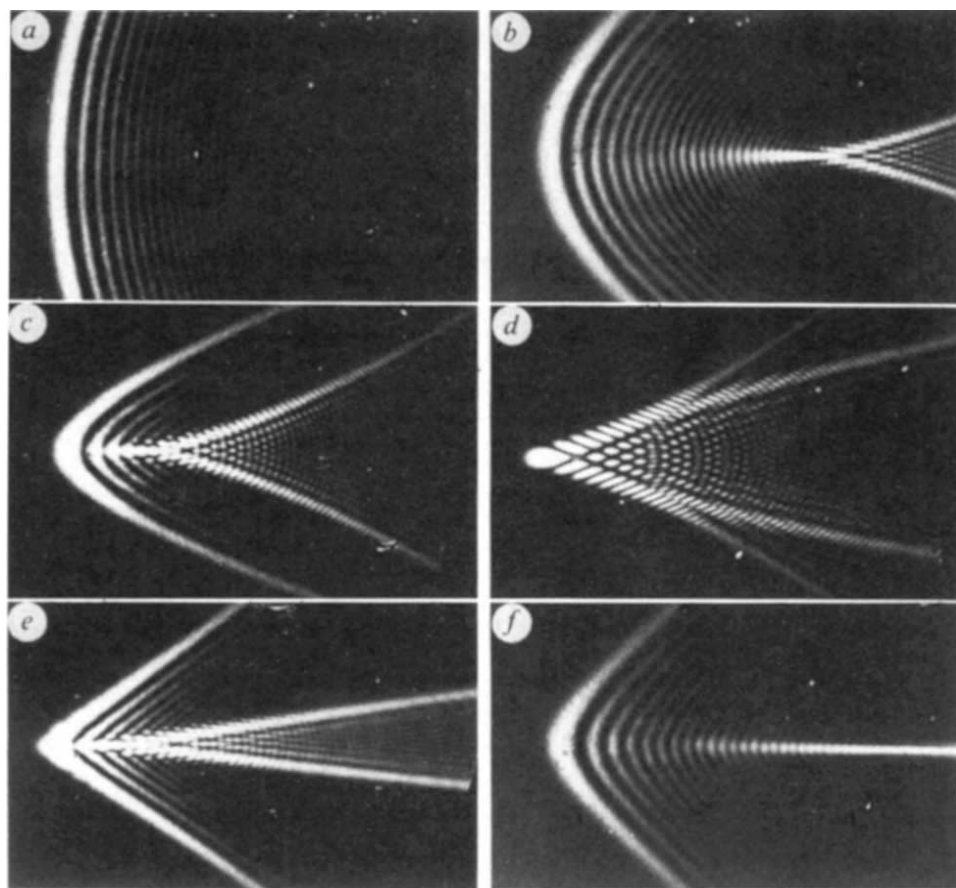


Fig. 4 Photographs of rainbow region scattering patterns arranged in order of increasing drop axis ratio D/H . The horizontal scattering angle θ increases from left to right. The cusp in *b* moved into view from the right as D/H was increased. *d* is the focal section of a hyperbolic-umbilic diffraction catastrophe while *c* and *e* manifest the unfolding of the catastrophe in response to small changes in D/H . Scale bar, 5°.

catastrophes. Consequently, the normal form^{1,2} of the associated diffraction integral has the control parameter $C_3 = 0$. Evidently the transverse control parameters C_1 and C_2 are locally related to β and $\theta - \theta_R$ by a linear transformation. The parallelogram structure is sheared with an apex angle $\approx 43.5 \pm 1^\circ$. Figure 4*b*, *c*, *e* and *f* correspond to sheared and scaled sections of D_4^+ patterns having $C_3 \neq 0$. Inspection of the patterns of the elementary catastrophes^{1,4} confirms that only the D_4^+ describes our observations.

When $C_3 \neq 0$, the D_4^+ pattern may be partitioned into three angular regions according to the number of stationary points of the phase function within the diffraction integral^{1,5,6}. The number of points corresponds to the number of once-reflected (twice refracted) rays which contribute to the scattering. This partitioning is shown in Fig. 5*a* for the pattern in Fig. 4*c*. That the right-most region has four rays was confirmed by direct observation. A camera having a small aperture, positioned as shown in Fig. 2, was focused on the drop. Figure 5*b* is drawn from a representative image so as to show the locations of the four observed rays. Rays 1 and 2 lie in plane *P* and correspond to those in Fig. 1. Rays 3 and 4 are horizontally directed, having $\beta \approx 0^\circ$ when outside the drop. They are skew rays because inside the drop they do not lie in a horizontal plane. These rays were not anticipated from previous studies^{13,14} of rainbows of spheroidal drops, which considered only rays confined to a plane.

Other features observed were consistent with those expected of a D_4^+ diffraction catastrophe at short wavelengths $\lambda \ll D$. The axis ratio D/H which gives $C_3 = 0$ should be independent of D for spheroidal drops having the same refractive index. To check this prediction, drops were illuminated with diffuse white light (see Fig. 2) and their profiles were photographed. Before each photograph, D/H was adjusted so that the laser scattering pattern was a focal section as in Fig. 4*d*. Photographs of six drops having D of 1.5–2.4 mm gave axis ratios not correlated with D . The average measured D/H of 1.305 ± 0.016 is a lower

limit of the true axis ratio because any misalignment of the camera's optical axis from the equatorial plane would give a systematic reduction in the measured D/H .

For scattering from large spherical drops, Airy's theory^{1,7} predicts that when $\theta = \theta_R$, the intensity I at a distance r has $I \propto (D/\lambda)^{1/3} (D/r)^2$. Examination of the D_4^+ diffraction integral^{1,5,6} gives $I \propto (D/\lambda)^{2/3} (D/r)^2$ when C_3 , β and $\theta - \theta_R$ all vanish. Visual inspections and photographs confirm that the scattering near the apex of the focal section appears brighter than that near θ_R of the other sections such as Fig. 4*b* and *c*. This focusing for $C_3 \approx 0$ may be observable in nature when the Sun is close to the horizon. Raindrops having $D \approx 4$ mm have an axis ratio ≈ 1.3 . However, such large drops are flattened below the centre¹⁵ and the effect on the focusing is not known.

These experiments were carried out while P.M. was at the Jet Propulsion Laboratory on a sabbatical leave. The research was partially supported by NASA and by the Office of Naval Research.

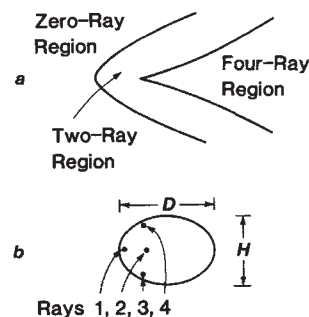


Fig. 5 *a*, Partitioning of the angular regions implied by the theory of hyperbolic-umbilic diffraction catastrophes for sections having $C_3 \neq 0$. *b*, Ray locations evident from the patches in Fig. 3. The camera's aperture was in the four-ray region.

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Rainbow scattering from spheroidal drops—an explanation of the hyperbolic umbilic foci

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An experiment by Marston and Trinh¹, showed how an oblate spheroidal water drop illuminated by a parallel beam of light forms a caustic in the far field characterized by the hyperbolic umbilic catastrophe—a generalization of the primary rainbow formed by a spherical drop. As the axis-ratio of the drop was changed, they observed a singular section of the catastrophe appearing for $D/H = 1.305 \pm 0.016$ — D being the diameter in the horizontal equatorial plane and H the diameter along the vertical axis of rotational symmetry. I show here how this ratio may be calculated to confirm quantitatively the arguments² that the hyperbolic-umbilic catastrophe is associated with skew rays, and predict the occurrence of novel 'lips' phenomena, yet to be observed.

PQRQ'P' is the critical (Descartes) ray in the equatorial plane (Fig. 1). Keeping the incident direction fixed, if we were to move the entry point away from Q, but still in the equatorial plane, standard rainbow theory would then tell us that the resulting angle of deviation is a minimum for entry at Q—the angles of incidence, i , and refraction, r , are given by $\sin i = \sqrt{\{(4 - \mu^2)/3\}}$, $\sin r = \sqrt{\{(4 - \mu^2)/3\}}$ and are connected by Snell's law $\sin i = \mu \sin r$, where μ is the refractive index.

If we now vary the entry point in the perpendicular (vertical) plane, still keeping the incident direction fixed, the once-refracted ray will, in general, pass above or below R by a first-order amount. However, we can show that, for a critical value of D/H , it continues to pass through R, to first order, and therefore emerges (by symmetry) displaced from the equatorial plane but without change of deviation, to first order. That is, we have directional focusing in the vertical as well as in the horizontal plane. Such simultaneous focusing in two directions gives rise to what is called, in catastrophe optics, an umbilic caustic. At R there is a horizontal focal line.

To find the critical D/H take the axes as shown, let the point of entry move vertically by ϵ , and assume that the refracted ray passes through R. To first order the direction cosines of the incident ray, the normal to the surface and the refracted ray are the rows of the determinant

$$\begin{vmatrix} \sin i & 0 & \cos i \\ 0 & \epsilon/\rho & 1 \\ \sin r & \epsilon/s & \cos r \end{vmatrix}$$

where $s = QR = D \cos r$ and ρ is the radius of curvature of the surface in the vertical plane, $\frac{1}{2}H^2/D$.

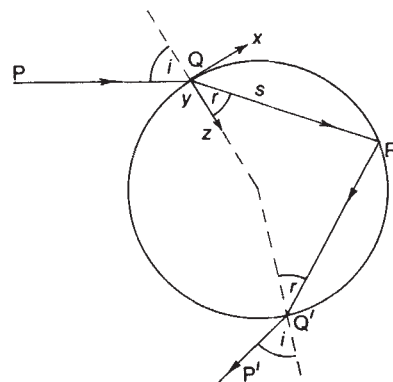


Fig. 1 The equatorial (horizontal) section of a spheroidal water drop showing the critical ray of minimum deviation.

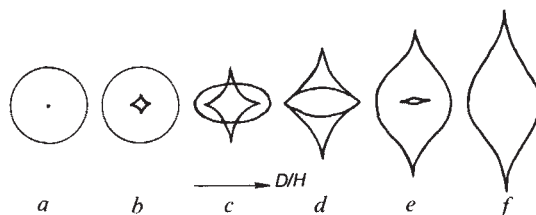


Fig. 2 Sequence of caustics in the far field as D/H increases from 1. a, The circular rainbow with a singular point at its centre. On perturbation the point breaks up (b, c) into an expanding four-cusped figure. At d, two hyperbolic umbilic foci occur. On further increase of D/H , the inner figure contracts (e), and then disappears (f) in a lips event. The angular width of the complete figure is the same throughout.

Snell's law is satisfied to first order without further conditions, but for the three directions to be coplanar the above determinant must be zero. A short calculation then yields the surprisingly simple result

$$\frac{D}{H} = \sec r = \sqrt{\frac{3\mu^2}{4(\mu^2 - 1)}}$$

Putting $\mu = 1.3317$, the refractive index of water at the wavelength used in the experiments ($\lambda = 633$ nm), gives $D/H = 1.3114$, which agrees with the observed value of 1.305 ± 0.016 . This is a stringent test that the shape of the drop is spheroidal.

I now show, using the principles of catastrophe optics, that the global topology of the caustic is that sketched in Fig. 2, taken from Hannay's work in appendix 2 of ref. 2. First notice that as $\mu \rightarrow 2$, the above critical ratio $D/H \rightarrow 1$ and that both i and r approach zero. That is, the caustic structure collapses into a single point in direction space (backwards)—the perfect 'cat's-eye' reflector. Now unfold (that is, perturb) this degenerate focus by treating $(2 - \mu)$ and D/H as control variables; keeping $D/H = 1$ and varying μ from 2 causes the familiar rainbow circle to grow from the central point (Fig 2a). Now keep μ fixed and increase D/H : the sequence is then Fig 2a to f with two hyperbolic umbilics appearing symmetrically in the equatorial plane in Fig 2d. These are the ones calculated above.

The lips event occurring between Fig. 2e and f is at $D/H = \sqrt{\{\mu/2(\mu - 1)\}} = 1.4168$ for the above value of μ . (There appears to be a second horizontal lips event on the axis at $D/H = \sqrt{\{(2\mu - 1)/2(\mu - 1)\}} = 1.5835$, not part of this catastrophe and arising from rays that pass through the centre of vertical curvature of the far side of the drop.)

Equation (A2.2) in ref. 2 shows that the form of the wavefront that emerges from the drop in the most singular case (spherical drop with $\mu = 2$), specified by its deviation $f(x, y)$ from a plane, is, to lowest order, $f = A(x^4 + 2x^2y^2 + y^4)$, which is a singularity of infinite codimension; that is, there are an infinite number of unfolding terms. However, the symmetry-breaking perturbation

from sphere to spheroid, with $\mu \neq 2$, yields a structurally stable caustic.

To establish directly that the umbilics calculated above are indeed hyperbolic rather than elliptic would have required a complicated analysis of the cubic terms in the expression for the wavefront as it was refracted and reflected astigmatically through the spheroidal water drop. But recognizing that the umbilics are part of the unfolding of a higher catastrophe removes the need for this calculation, for there is no reason to suspect that they change their character when the unfolding is finite rather than infinitesimal, as assumed in the theory. Note, however, that I have calculated the critical D/H ratios exactly, rather than merely to lowest order in $2-\mu$ and $D/H-1$.

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A very narrow Mössbauer resonance in ^{63}Ni

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Investigations of the Mössbauer effect in nickel have been less fruitful than those on iron, because until now there has been no convenient resonance akin to the 14-keV transition (half-life 98 ns) in ^{57}Fe . So far the only available Mössbauer resonance in nickel has been the 67-keV transition (half-life, 5 ns) in ^{61}Ni , whose natural resonance line is so broad that its width is comparable to the nuclear Zeeman splitting in ferromagnetic nickel metal. The result is a spectrum comprising a single broad line instead of a well-resolved splitting into discrete lines, as is the case for narrow resonances. We have recently succeeded in a scattering experiment in observing the Mössbauer effect in the 87-keV transition (half-life 1.7 μs) in radioactive ^{63}Ni ($t_{1/2} = 100$ yr). Because of the long lifetime of this state, its natural linewidth is smaller than that of ^{61}Ni ($\times 330$), and the commonly used ^{57}Fe ($\times 17$), but still larger than that of ^{67}Zn ($\times 0.2$), which has the highest relative energy resolution of all Mössbauer resonances in use. The use of ^{63}Ni should thus permit us in future to perform detailed Mössbauer studies in nickel.

The γ -source for observing the Mössbauer effect in ^{63}Ni is ^{63}Co (half-life 27.5-s), produced by the $^{64}\text{Ni}(t, \alpha)^{63}\text{Co}$ reaction with the triton beam of the Technical University of Munich Compact Cyclotron¹. With this reaction, ^{63}Co could be produced essentially free of unwanted radioactivity, which is not the case when using other projectiles. A production rate for ^{63}Co of ~ 1 mCi min^{-1} was possible during irradiation with a triton beam of ~ 2 μA .

A total of 15 targets were used to avoid accumulation of long-lived activity by repeated irradiation. The target material was metallic nickel foil of 27 mg cm^{-2} thickness and 10 mm diameter, made of enriched (97.7%) ^{64}Ni . These foils were soldered to coin-shaped target holders made of copper disks 6 mm thick and 25 mm in diameter. A hole of 10-mm diameter was bored along the symmetry axis of the discs, leaving a wall-thickness of 0.5 mm at the bottom. The ^{64}Ni foils were soldered using lead solder into this cylindrical cavity under vacuum conditions. Before irradiation the targets were cooled to liquid nitrogen temperature. Because of the energy loss of the tritons in the target foil, we are sure that the temperature of the ^{64}Ni is higher than 77 K during irradiation presumably allowing annealing of radiation damage in the nickel lattice to

occur. After irradiation, the heat dissipated in the nickel foil is absorbed owing to the high heat capacity of copper target holder, thus rapidly cooling the target foil again to the temperature of liquid nitrogen. The sources were then quickly transported, within 6–8 s, to a measuring position, well-shielded from the accelerator. This was accomplished by rolling the targets through inclined tubes of rectangular cross-section. The absorber and scatterer was a ^{63}Ni foil (12% ^{63}Ni , 15-mm diameter, 0.2-mm thickness), which was also cooled to liquid nitrogen temperature.

We have used 41 mg of ^{63}Ni corresponding to 2.3 Ci of the 100-yr half-life activity. Ferromagnetic nickel metal was chosen as the chemical form of source and absorber (scatterer), and both are thus subject to a magnetic nuclear Zeeman splitting. Only the unshifted central resonance line was covered by the velocity drive. The relative Doppler velocity between the source and the absorber (scatterer) was obtained by moving the absorber sinusoidally with a piezo-drive. The maximum velocity was 35 $\mu\text{m s}^{-1}$ and the velocity drive was calibrated by observing the well-known isomer shift between $^{67}\text{GaCu}$ and $^{67}\text{Zn-}\beta$ -brass. The scattered and transmitted radiation of the sources were measured simultaneously by two planar intrinsic germanium detectors and recorded by two multichannel-analysers synchronously with the velocity of the drive.

After more than 200 measuring cycles (altogether 10 h of measuring time) a resonance line was seen in the scattering spectrum with a linewidth of 5 times the minimum resonance width, having taken the scatterer thickness into account. This spectrum is shown in Fig. 1. Its intensity corresponded very well

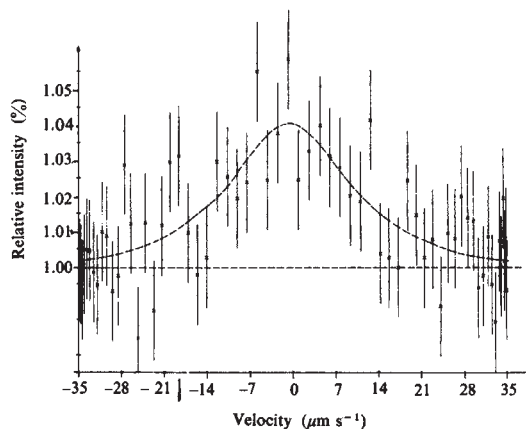


Fig. 1 Intensity of the recoil-less scattered radiation from a ^{63}Ni scatterer. (Only the central resonance line is observed.)

with the value calculated by applying to the data the procedure of Debrunner and Morrison². This indicates that the adopted Debye-Waller factor for liquid nitrogen temperature, derived from ^{61}Ni experiments, reproduces the actual intensity very well. The resonance line disappeared when the temperature of source and scatterer was raised to room temperature. However, the statistics were still not good enough to observe the resonance in transmission geometry. The broadening of the line is most probably due to radiation damage in the source material.

We shall now try to reduce the linewidth and find a compound that gives an unsplit single emission line without generating too much unwanted radioactivity. Also certain basic experiments, such as the determination of the ground state magnetic moment of ^{63}Ni , are being planned.

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Structure of Theta-1, the first unidimensional medium-pore high-silica zeolite

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Synthetic high-silica zeolites (typified by ZSM-5) are powerful catalysts used extensively in the petroleum industry. The channels and cavities of their aluminosilicate framework render their catalytic activity very shape-specific. The varying attributes of individual members of this family depend primarily on subtle changes in framework topology. Unfortunately, these high-silica zeolites are difficult to synthesize as large, that is $>100\ \mu\text{m}$, single crystals, so conventional X-ray structural methods are not appropriate. We have used a combination of techniques to elucidate the framework topology of a new, synthetic high-silica zeolite, Theta-1, which was first prepared as small $<2\ \mu\text{m}$ crystals in our laboratories. It exhibits a novel unidimensional 10-T-ring channel system with a medium pore structure, thereby differing from other known structure-types that classify zeolites and suggesting unique shape-selective properties.

Crystals of Theta-1 were synthesized as described previously^{1,2} and exhibited rod-like morphology, being $\sim 0.1\ \mu\text{m}$ in diameter and $\sim 2\ \mu\text{m}$ in length. For characterization, samples were converted to the hydrogen form using standard methods^{1,2}. The bulk $\text{SiO}_2:\text{Al}_2\text{O}_3$ molar ratio was determined by X-ray fluorescence

indexed on the basis of a C-centred orthorhombic unit cell (uc) giving the dimensions $a = 13.836 \pm 0.003$, $b = 17.415(4)$ and $c = 5.042(1)\ \text{\AA}$ by refining 28 calibrated and measured lines.

Structural analysis followed these multi-technique data. A framework density of 20.5 tetrahedral-atoms (T-atoms) per $1,000\ \text{\AA}^3$ was calculated⁶ from the crystal density of $2.05\ \text{g cm}^{-3}$ measured by mesitylene displacement. Adsorption (Table 1) showed a maximum void fraction (V_f) 0.21 which also gave⁶ 20.5 T-atoms per $1,000\ \text{\AA}^3$. These translated to 24.9 T-atoms per uc in good agreement with a minimum site multiplicity of 4 (that is 24 T-atoms per uc) for each of the three space groups⁷. Trial structures for Theta-1 were evaluated by generating sufficient possible Fourier projections in the a - b plane from experimentally-measured integrated intensities. (Such a method is only possible for centrosymmetric projections down short axes, such as the $5.04\text{-}\text{\AA}$ axis of Theta-1, where atomic overlap is minimized.) Full-matrix least-squares refinement of one trial structure having the Cmc2₁ space group successfully converged to $R = 0.13$ for 302 reflections ($2\theta \leq 90^\circ$). The framework topology of this structure is shown in Fig. 1A, and the refined atomic coordinates are listed in Table 2. The mean of the seven independent Si-Si distances was $3.10(7)\ \text{\AA}$ in a range $3.01\text{--}3.24\ \text{\AA}$ with all distances lying within the 2σ limit. The SiO_4 tetrahedral geometry was suitable. The range of 14 independent Si-O distances was between 1.50 and $1.65\ \text{\AA}$ with a mean of $1.59(6)\ \text{\AA}$. The mean O-Si-O tetrahedral angle was $109(4)^\circ$ in the range $102\text{--}118^\circ$.

The proposed structure is rich in 5-T rings, as is expected for high-silica tectosilicate structures⁵, and can be constructed from complex 5-1 secondary building units  (from ref. 8). This places the proposed structure in the Mordenite (Pentasil) group

Table 1 Adsorption properties of Theta-1

Adsorbate	Kinetic diameter ($\text{\AA}$)	V_p ($\text{cm}^3\ \text{g}^{-1}$)	V_f
N_2	3.64	0.1	0.21
H_2O	2.65	0.058	0.12
<i>n</i> -hexane	4.3	0.089	0.18
<i>p</i> -xylene	5.85	0.062	0.13
Cyclohexane	6.0	0.021	0.04
<i>m</i> -xylene	6.0	0.031	0.06

Void volume V_p ($\text{cm}^3\ \text{g}^{-1}$) is the total micropore volume in hydrogen form of Theta-1, calculated using normal liquid density at adsorption temperature. The void fraction, $V_f = V_p$ ($\text{cm}^3\ \text{g}^{-1}$) $\times d_c$ (g cm^{-3}), where d_c ($=2.05\ \text{g cm}^{-3}$) is the measured crystal density. All samples activated by calcination at 550°C for 1 h. Adsorption measurements by microbalance techniques at room temperature with relative pressure $P/P_0 = 0.5$. N_2 adsorption by single point Brunauer-Emmett-Teller test, -183°C $P/P_0 = 0.9$.

(XRF) and confirmed by magic angle spinning NMR (MASNMR)³. Typical XRF values were 60:1. ^{29}Si MASNMR spectra showed peaks of relative area 13.9:1 at -113 and -105 p.p.m. relative to tetramethylsilane, confirming^{4,5} a tecto-aluminosilicate with a 60:1 $\text{SiO}_2:\text{Al}_2\text{O}_3$ ratio. This was supported by a single ^{27}Al peak at ~ 57 p.p.m. relative to $\text{Al}(\text{H}_2\text{O})_6^{3+}$, indicative of tetrahedral Al. The adsorption properties of Theta-1 were measured with a range of adsorbates to probe shape-selectivity (Table 1). The results show that the zeolite is microporous and that access to the pore structure becomes restricted at $6\ \text{\AA}$.

The structure of Theta-1 was studied by the diffraction of both electrons and X rays. Selected-area electron diffraction patterns show orthorhombic symmetry and reveal unit cell parameters with a short c -axis repeat value of $5\ \text{\AA}$ which is particularly significant in zeolites. In addition, systematic absences are consistent with three possible space groups, namely Cmc₂, C2cm and Cmc2₁. The X-ray powder diffraction pattern was

Table 2 Fractional atomic coordinates ($\times 10^3$)

Atom	x	y	z
Si(1)	283.8 (9)	50.3 (7)	250*
Si(2)	201 (1)	215.7 (8)	312 (4)
Si(3)	500†	218 (1)	824 (6)
Si(4)	500†	118 (1)	320 (7)
O(11)	264 (2)	-10 (2)	493 (8)
O(12)	214 (2)	125 (2)	309 (8)
O(14)	404 (2)	65 (1)	280 (9)
O(22)	257 (3)	257 (2)	534 (7)
O(23)	407 (2)	269 (2)	795 (10)
O(34)	500*	152 (3)	628 (10)
O(43)	500*	187 (2)	1,132 (9)

* Figures in parentheses give $\pm$ s.d.

† Fixed parameter.

of zeolite structures⁵. These include mordenite, ferrierite, dachiardite, epistilbite and bikitaite (ZSM-5 and ZSM-11). A projection in the a - b plane shows zig-zag chains of edge-shared 5-5-T-rings (Fig. 1B) in the a direction linked by 6-T-rings in the b direction to form elliptical 10-T-ring channels running along the c direction. The same structural feature is found in ferrierite, ZSM-5 and ZSM-11 (ref. 9), although with different specific topology. In contrast, in the c direction, projections strongly resemble comparable projections in bikitaite^{5,10} and consist of interlinked 6-T-ring sheets. Thus, like bikitaite, the proposed topology has a unidimensional channel system. The presence of 5-T-rings in Theta-1 is confirmed by IR spectroscopy. A band at $1,230\text{--}1,210\ \text{cm}^{-1}$ has previously¹¹ been assigned to this structural arrangement.

V_f values for adsorbate molecules of different size and shape (Table 1) indicate a pore diameter of $\sim 6\ \text{\AA}$ similar to that found for ZSM-5. However, ZSM-5 has two intersecting 10-T-ring structural channels and a maximum V_f of 0.32. The much lower value of 0.22 for Theta-1 argues for a unidimensional 10-T-ring channel system, also made necessary by the very short c -axis

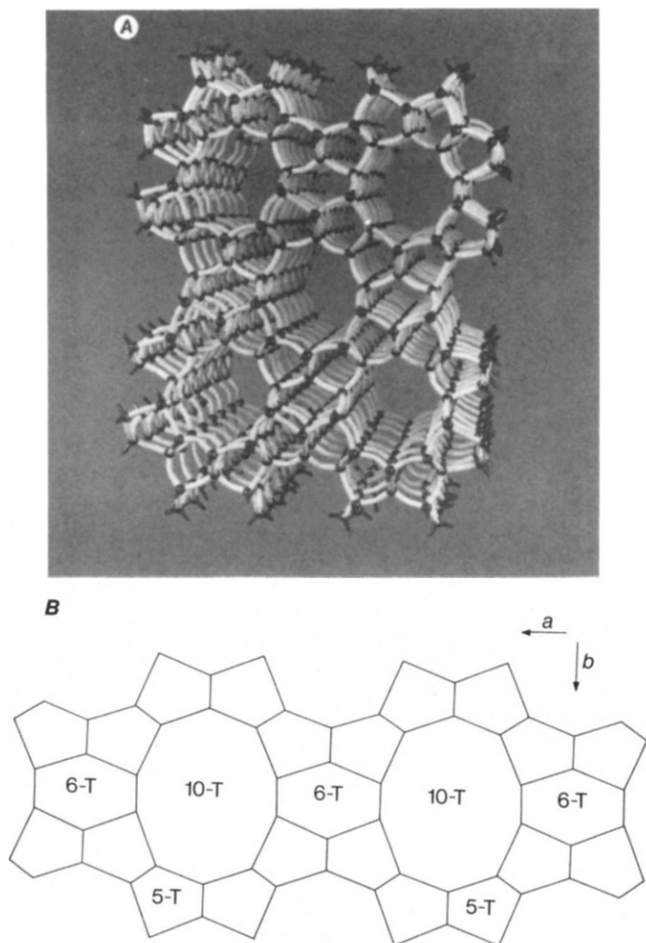


Fig. 1 A, Theta-1 framework viewed down axis *c*. This projection is similar to the *a-c* projection in the zeolite Bikitaite. B, Projection in the *a-b* plane of 5-5-T and 6-T rings to show the elliptical 10-T ring channels in the *c* direction.

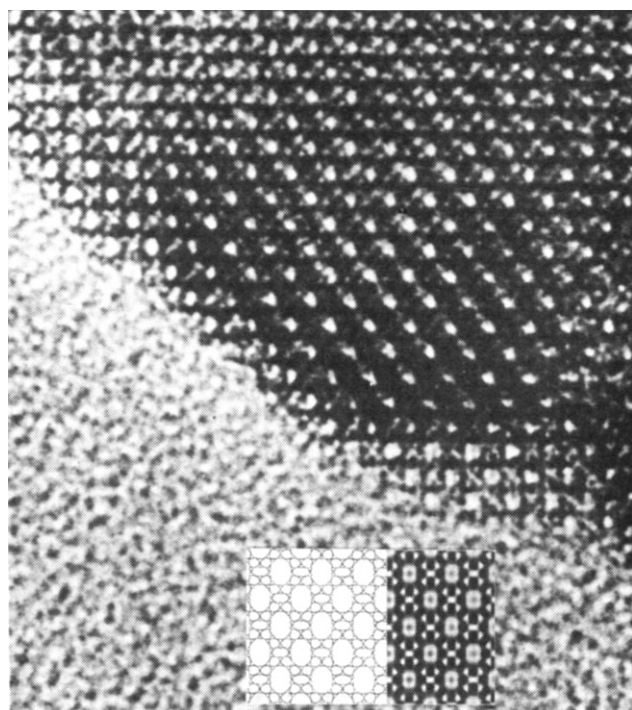


Fig. 2 High-resolution transmission electron micrograph in the *a-b* projection. Insets show schematic proposed structure (left) and calculated image (right).

(5.042 Å). The ^{29}Si MASNMR of a Theta-1 aluminosilicate framework, dealuminated by calcination at 800 °C, showed closely positioned Si(OAl) peaks at -110.5, -112.4 and -113.9 p.p.m. The broad width of the peak at -110.5 p.p.m. (2.5 compared with 1.9 p.p.m.) strongly suggests a composite of two peaks at very similar chemical shifts, or a minimum of 3 or 4 crystallographically-distinct T-atoms. Using the relative peak areas, framework density, and space-group site multiplicity restrictions³, we obtained relative site occupancies of 8:8:8 or 4:4:8:8 per uc. The proposed structure has four crystallographically-distinct T-atoms of relative occupancy 4:4:8:8. The two four-occupied sites are topologically very similar and would be expected to have very similar chemical shifts.

Direct evidence for the structural arrangement in the *a-b* projection (viewing down the main channels) has been obtained by high-resolution transmission electron microscopy, as shown in Fig. 2. The schematic projection for the proposed structure (oxygen atoms omitted for clarity) is inset, together with a theoretical image, computed for specimen thickness, operational parameters and imaging defects of the microscope¹².

The proposed structure of Theta-1 is the first example of a new topologically-distinct structure-type. In accordance with IUPAC recommendations used to classify zeolites and related tectosilicates⁹, we propose to designate this new structure type as TON. The high-silica content and the 10-T-ring channels give Theta-1 aluminosilicate similar properties to other medium-pore high-silica zeolites such as ZSM-5, ZSM-11 and ferrierite. However, molecular shape selective catalysis in zeolites is highly dependent on the geometry and tortuosity of the channel and cavity systems¹³. Matching the geometries of these channels and cavities with those of the reactants, products and transition states in a catalytic reaction gives insight into the zeolite catalytic activity and selectivity. Thus, the unique high-silica unidimensional 10-T-ring channel system, particularly the lack of channel intersections and associated 'cage' volumes¹⁴, should give Theta-1 valuable structure-specific properties. The natural zeolite Laumontite¹⁰ also has a unidimensional 10-T-ring structure. However, it is a low-silica zeolite ($\text{SiO}_2:\text{Al}_2\text{O}_3$ range 3.5-4.56) and thus can be expected to have a low thermal stability in the hydrogen-form for many of the catalytic and separation applications potentially feasible for Theta-1.

Recently, several novel zeolites have been reported; Nu-10 (ref. 15), KZ-2 (ref. 16), ISI-1 (ref. 17) and ZSM-22 (ref. 18). Their X-ray powder diffraction patterns are consistent with their also having the TON structure type.

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The α -recoil effects of uranium in the Oklo reactor

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Until recently, it was thought that chemical elements were synthesized only in stars. The discovery of the Oklo phenomenon¹⁻⁶ in 1972 revealed, however, that there was a nuclear 'fire' on the Earth 1,700 Myr ago. We have now carried out a series of acid-leaching experiments on a sample of uranium ore from reactor zone no. 10 of the Oklo mines in Gabon, Africa, and observe anomalously high $^{234}\text{U}/^{238}\text{U}$ ratios accompanied by modestly increased $^{235}\text{U}/^{238}\text{U}$ ratios in uranium fractions. These results, which can be interpreted as being due to the α -recoil effects of ^{238}U and ^{239}Pu , provide a convenient way of calculating the conversion factor (or the fraction of uranium atoms converted to plutonium) of the natural reactors from radiochemical data, obviating the necessity for mass-spectrometric measurements.

Almost 30 years ago, Cherdyn'tsev⁷⁻⁹ discovered that the $^{234}\text{U}/^{238}\text{U}$ ratio does not remain constant in a mineral extract: for example, uranium in a zircon extract showed a $^{234}\text{U}/^{238}\text{U}$ ratio of 1.8 (Ci/Ci) while that of surface waters was sometimes as high as 7-8. Kigoshi¹⁰ proposed that the excess ^{234}U may come from α -recoil ^{234}Th atoms ejected into the groundwater. Kobashi *et al.*^{11,12} have carried out a series of leaching experiments with the radioactive minerals euxenite, uraninite and samarskite. Their results indicate that the observed isotopic fractionations of uranium, thorium and radium can be attributed to the difference in physicochemical state between the isotopes in the minerals and can be explained in terms of: (1) damage due to α -recoil displacement; (2) annealing; and (3) the difference in oxidation states between ^{238}U and ^{234}U . Anomalously high $^{234}\text{U}/^{238}\text{U}$ ratios are sometimes found in secondary minerals containing uranium. For example, Sakanoue and Komura¹³ found a value of ~ 15 for a sample of maghaemite from the Abeshiro mine in Aomori Prefecture, Japan. Detailed studies on the nature of α -recoil and preferential solution effects have recently been carried out by Fleischer and co-workers¹⁴⁻¹⁶ using the fission track counting method. We used 1.089-g sample of powdered uranium ore from reactor zone 10 of the Oklo uranium deposit in the Republic of Gabon, Africa. The uranium content of this ore sample, which was a steel-grey colour, was 34.5%

and the $^{235}\text{U}/^{238}\text{U}$ ratio was 0.660 atom %. It was leached in a series of experiments, beginning with 40 cm³ of 0.1 M HCl, and was subsequently treated successively with 6 M HCl, 9 M HCl and 8 M HNO₃. The first passage of the 8 M HNO₃ solution through the column resulted in a marked increase in the rate of reaction. When this step was completed, the residue was transferred to a small beaker and treated with 8 M HNO₃ for ~ 3 h at near boiling point. The residue was filtered and washed with 8 M HNO₃. The little residue which remained after this was transferred to a Teflon beaker and treated with concentrated HF.

The radiochemical procedures used to separate and purify the uranium were essentially those used by Sakuragi *et al.*¹⁷. The α -spectrometry was carried out with an ORTEC-576 α -spectrometer with dual silicon surface barrier detectors (ORTEC BR-21-300-100) attached to a multiplexer/router (ORTEC-476) and a multichannel analyser (Norland Ino-Tech IT-5300). The FWHM for the ^{234}U and ^{238}U peaks is ~ 40 -50 keV. Because α energies of ^{226}Ra (4.785 MeV) and ^{230}Th (4.684 MeV) are close to that of ^{234}U (4.773 MeV), we made sure that thorium and radium had been completely removed from the purified uranium fraction.

Table 1 shows the experimental results. Figure 1 compares the α spectra of fractions 4 and 10. The most remarkable feature of our results is the extremely high $^{234}\text{U}/^{238}\text{U}$ ratio of the uranium fraction from leaching step 10 (64.3 ± 3.0), which is far greater than the values reported by previous investigators with various radioactive minerals^{9,11,12}.

Most of the uranium in the ore sample had been dissolved in the acid solutions by the time the 8 M HNO₃ treatment was completed and leachate fraction 7 was obtained. The observed $^{234}\text{U}/^{238}\text{U}$ ratios in leachates 3-7 were essentially the same as the radioactive equilibrium value of 1.00. The marked increases observed in the $^{234}\text{U}/^{238}\text{U}$ ratios in fractions 8-11 may be due to the fact that we are observing a preferential solution effect of recoil atoms of ^{234}U present in mineral phases whose uranium content was much lower than that of the reactor core material. The Oklo ore sample studied here contained tiny inclusions of haematite, clay and breccia. These inclusions, which contained very little uranium initially, may have been accumulating recoil atoms of ^{234}U over many years. The ^{234}U atoms which entered the crystal lattices of the mineral inclusions would be loosely held and could be leached by the acid solutions much more easily than any ^{235}U and ^{238}U originally present. Alternatively, if the effects are due to emplacement of recoiling nuclei into a second, relatively low-uranium phase adjacent to the dominant uranium-rich mineral, direct dissolution of the surface layers would remove the implanted atoms and so produce the observed effect.

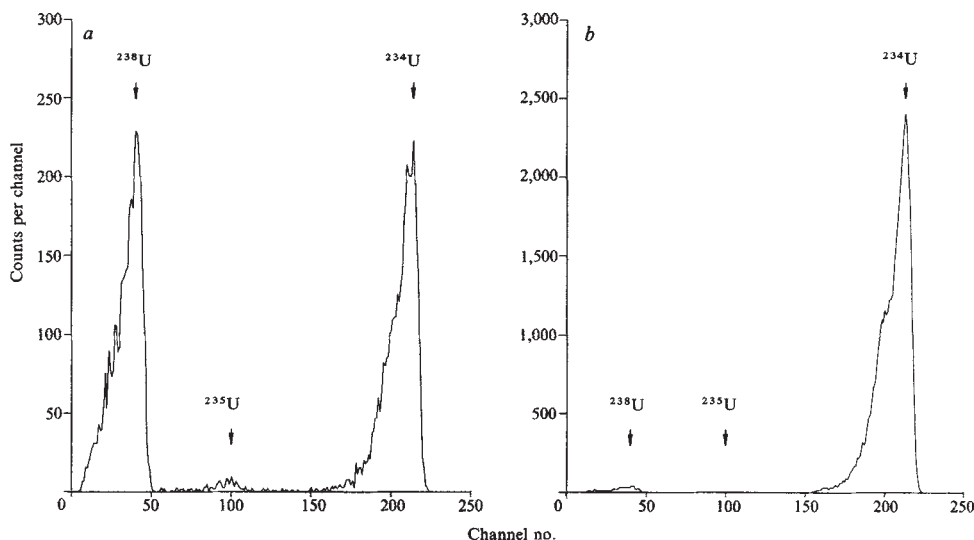


Fig. 1 The α spectra of uranium fractions: a, from fraction 4 obtained after treating with 200 cm³ of 6 M HCl; b, from fraction 10 obtained after treating with 50 cm³ of hot 8 M HNO₃.

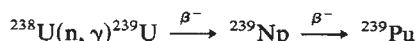
Table 1 Variation of the isotopic composition of uranium extracted from a sample of Oklo ore deposit in the acid-leaching experiments

Fraction no.	Acid used	Amount of U leached (mg)	$^{234}\text{U}/^{238}\text{U}$ (Ci/Ci)	$^{235}\text{U}/^{238}\text{U}$ (Ci/Ci)	Notes
1	0.1 M HCl*	70.6	1.15 ± 0.03	0.035 ± 0.004	Leached with 40 cm ³ of acid for 1 day
2	0.1 M HCl†		1.22 ± 0.02	0.037 ± 0.003	
3	6 M HCl*		0.97 ± 0.02	0.039 ± 0.003	
4	6 M HCl†	110.3	1.02 ± 0.03	0.036 ± 0.004	Leached with 200 cm ³ of acid for several days
5	9 M HCl*		1.03 ± 0.05	0.035 ± 0.006	
6	9 M HCl†		1.01 ± 0.03	0.036 ± 0.005	
7	8 M HNO ₃ *	91.8	1.01 ± 0.04	0.045 ± 0.007	Leached with 40 cm ³ of acid for 2-3 h
8	8 M HNO ₃ ‡		4.14 ± 0.28	0.051 ± 0.014	
9	8 M HNO ₃ §		5.47 ± 0.42	0.065 ± 0.019	
10	8 M HMO ₃ (hot)	12.3×10^{-3}	64.3 ± 3.0	0.120 ± 0.017	Leached with 50 cm ³ of acid; heated for about 3 h
11	Conc. HF (hot)	11.1×10^{-3}	21.02 ± 0.51	0.064 ± 0.006	Leached with 50 cm ³ of acid; the residue was completely brought into solution

The sample was packed in a plastic column of a diameter 1 cm attached to a glass container into which a certain volume of acid solution was introduced. The acid solution was allowed to flow through the column containing the uranium ore sample at a slow rate and collected in a small beaker or a test tube. Only a small volume of leachate solution, which was collected at the beginning and at the end of each leaching step, was analysed for the uranium isotopes. A pressure of ~0.5-1.5 m (water-equivalent) was applied to the leaching column to speed up the passage of the acid solutions through the ore sample.

*Initial fraction. †Final fraction. ‡Final fraction 1. §Final fraction 2.

A marked increase in the $^{234}\text{U}/^{238}\text{U}$ ratio observed in leachate fractions 8-11 is accompanied by a modest increase in the $^{235}\text{U}/^{238}\text{U}$ ratios (Table 1, Fig. 2). The observed increase in the $^{235}\text{U}/^{238}\text{U}$ ratio in some of the leachate fractions of the Oklo ore samples is to be expected, because the reactions

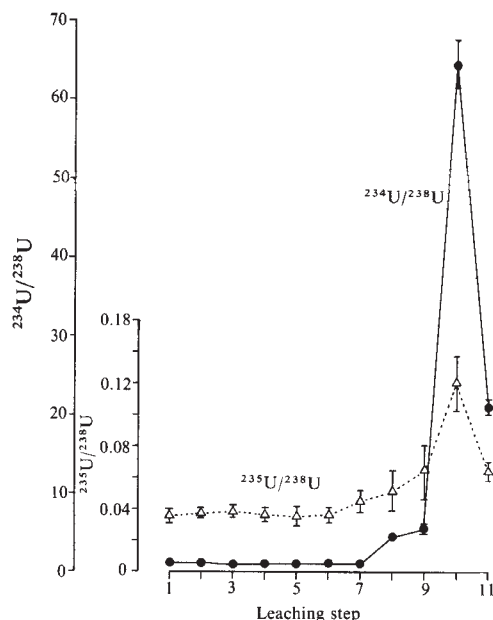


must have occurred on a large scale when the Oklo reactors were in operation. The α -recoil of the decaying ^{239}Pu atoms may have implanted the atoms of ^{235}U in the crystal lattices of nearby mineral inclusions.

The experimental data obtained here can be used to calculate the values of conversion factor C for the natural reactors, which can be defined as

$$\begin{aligned} \frac{dN_{235}}{dt} &= -N_{235}\sigma_{235}(1-C)\Phi \\ &= -N_{235}\sigma_{235}\Phi + N_{239}\lambda_{239} \end{aligned} \quad (1)$$

where Φ is the neutron flux, σ_{235} is the neutron-absorption cross-section of ^{235}U and λ_{239} is the decay constant of ^{239}Pu .

**Fig. 2** Variation of the $^{234}\text{U}/^{238}\text{U}$ and $^{235}\text{U}/^{238}\text{U}$ ratios of uranium extracted from a sample of Oklo ore deposit.

Because the same relationship holds for the ^{235}U atoms (N_{235}^P) which were created by the decay of ^{239}Pu atoms, we also have

$$\frac{dN_{235}^P}{dt} = -N_{235}^P\sigma_{235}\Phi + N_{239}\lambda_{239} \quad (2)$$

and, solving equations (1) and (2), we have

$$\frac{1}{C} = \frac{\log(N_{235}/N_0)}{\log\left[1 - \left(\frac{N_{238}}{N_{235}}\right)\left(\frac{A_{235}^P}{A_{234}}\right)\left(\frac{T_{235}}{T_{238}}\right)\right]} + 1 \quad (3)$$

where A_{235}^P is the activity of recoiled ^{235}U atoms from ^{239}Pu , A_{234} is the activity of recoiled ^{234}U atoms from ^{238}U , T_{235} and T_{238} are the half lives of ^{235}U and ^{238}U , respectively, and N_{235}/N_0 is the ratio of the ^{235}U contents of the uranium in the natural reactor and in the normal uranium ore, respectively.

For the uranium sample from reactor zone 10 of the Oklo mines, we have $(N_{235}/N_0) = 0.00660/0.00725$, $(A_{235}^P/A_{234}) = (0.120-0.036)/(64.3-1.0) = 0.084/63.3$ (see the data shown in Table 1, fraction number 10) and equation (3) yields a value of $C = 0.26$. Similarly, the observed $^{234}\text{U}/^{238}\text{U}$ and $^{235}\text{U}/^{238}\text{U}$ ratios for fraction 11 yield a value of $C = 0.27$. Hageman *et al.*¹⁸ and Neuilly *et al.*¹⁹ have reported values of C ranging from 0.37 to 0.58 and from 0.12 to 0.69 for a number of Oklo samples which they studied, respectively.

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A new heteropolyacid with carbon as a heteroatom in a Keggin-like structure

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In the group of compounds called heteropolyacids based on heteropolyanions represented by the general formula $(M'_xM_yO_z)^{m-}$, where the host atom M is usually tungsten or molybdenum, the most familiar are the 12-heteropolyanions (or acids) in which $x:y = 1:12$ and the heteroatom M' is Si, Ge, Sn, P, As, or the like. Structural investigations¹⁻³ have shown that the anion has a cage-like structure known as the Keggin structure. Generally, 12-heteropolyacids with this structure are strongly acidic and are also remarkably stable; $H_3(PW_{12}O_{40}) \cdot nH_2O$, for example, can be solidified from solution. I have obtained a novel compound believed to be a kind of 12-heteropolyacid (to be termed dodecatungsto-carbonic acid) with carbon as the heteroatom. The constitution of the new material is supported by the results of infrared and Raman spectroscopy.

Tungsten carbide WC (commercial powder) was dissolved in a 15% hydrogen peroxide (H_2O_2) solution, giving a strongly acidic solution almost without colour. (Although the reaction of WC with H_2O_2 appears not previously to have been reported, reactions of Mo (metal) and MoO_3 with H_2O_2 are known.⁴) Excess H_2O_2 was removed by catalytic decomposition using a platinized net, and the small amount of carbon soot floating in the solution, probably caused by free carbon in the WC, was carefully removed using 0.6- μm filter paper. The clear solutions thus obtained were dried at 50 °C and at 100 °C to give yellow non-crystalline solids (solid I and solid II, respectively).

The amount of CO_2 evolved from solid II by heating at 650 °C in an oxygen stream was measured by gas chromatography. From the weight of the residual solid, which appeared to be pure WO_3 , and the amount of CO_2 evolved, the ratio C/W in solid II was determined to be 1:12.00 (with a standard error of 0.45). No significant differences of the ratio were found when solid II was heated in an O_2 or He stream, indicating that its carbon is not in elemental form.

Thermal decomposition of solid II was followed by thermogravimetric analysis (TG) and differential thermal analysis (DTA) in a dry air stream, and solid II was found to decompose below 700 °C (Fig. 1) in two steps, each accompanied by an endothermic peak. The sole exothermic peak at 450 °C, which is not accompanied by weight change, may represent a crystal

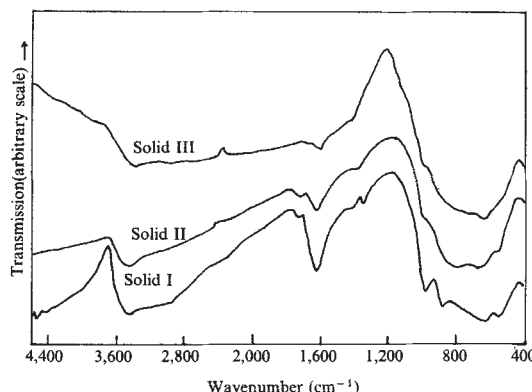


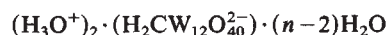
Fig. 1 Thermogravimetric (TG) and differential thermal analysis (DTA) for solid II. Initial sample weight, 72.1 mg; heating rate (T), 10 °C min^{-1} ; atmosphere, dry air (30 $cm^3 min^{-1}$).

phase change. Identical results were obtained in an Ar stream, showing that oxidation (for example, of CO) could not have been involved. The first endothermic step in the decomposition was shown to be dehydration by heat-treating solid II at 200 °C for 40 min, when CO_2 analysis of the material (solid III) revealed that the ratio C/W is again 1:12.

From the amount of weight loss in the second step, beginning at 280 °C, the empirical formula of the compound in the intermediate stage (solid III) is calculated to be $CO_2 \cdot 12WO_3 \cdot 4H_2O$ and, with the help of the TG data, the composition of solid II is found to be $CO_2 \cdot 12WO_3 \cdot 16H_2O$.

The infrared spectra (Fig. 2) of solids I, II and III each have a band at 1,730 cm^{-1} , although that in the spectrum of solid III is indistinct. The same vibrational frequency is characteristically found in heteropolyacids with anions of the Keggin type such as $H_3PMo_{12}O_{40} \cdot nH_2O$, and has been assigned to the frequency of pyramidal hydrated protons in groupings such as H_3O^+ (or $H_5O_2^+$) (see refs 5, 6).

These results suggest that the compound may be a type of 12-heteropolyacid with an anion whose atomic arrangement is the same as, or similar to, the Keggin structure, so that it may be written as $H_4(CW_{12}O_{40}) \cdot nH_2O$ or, more properly,



or



In an anion of Keggin-type incorporating P, for example, four W_3O_{13} units (each constructed from three WO_6 octahedra

Table 1 Vibrational frequencies of CO_3 group (cm^{-1})

Sample	Infrared/Raman	$\nu_1(A'_1)$	$\nu_2(A'_2)$	$\nu_3(E')$	$\nu_4(E')$
Solution I	Raman	960	890	ND	640
Solid I	Infrared	970	880	1,350, 1,400	640
	Raman	970	890	ND	630
Solid II	Infrared		870	1,400	650
	Raman	950		ND	630
Solid III	Infrared		(900)	1,400	640
	Raman	950		ND	(620)
$CaCO_3^*$	Infrared		879	1,429-1,492	706
Calcite	Raman	1,087		1,432	714
$CaCO_3^*$	Infrared	1,080	866	1,492, 1,504	706, 711
Aragonite	Raman	1,084	852	1,460	704
Selection rule	D_3	Infrared	—	+	+
		Raman	+	—	+
	C_s	Infrared/Raman	+	+	+(split)

ND, not detected; (), vague because of overlapping bands; +, active; —, inactive; *, vibrational frequencies of two types of $CaCO_3$ having different site symmetries (D_3 in calcite and C_s in aragonite) shown for comparison.

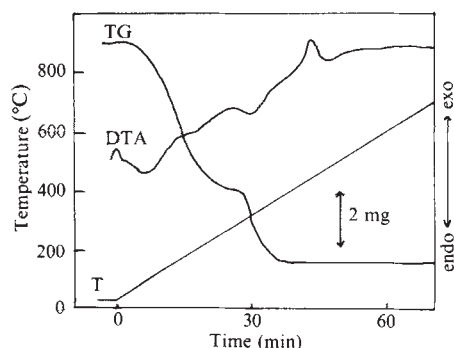


Fig. 2 Infrared spectra of solids I, II and III, recorded using the KBr pellet technique. Transmission has an arbitrary scale. Bands around $3,400$ and $1,620\text{ cm}^{-1}$ are due to H_2O in the solid states. Kinks around $2,400\text{ cm}^{-1}$ arise from the influence of CO_2 in the atmosphere.

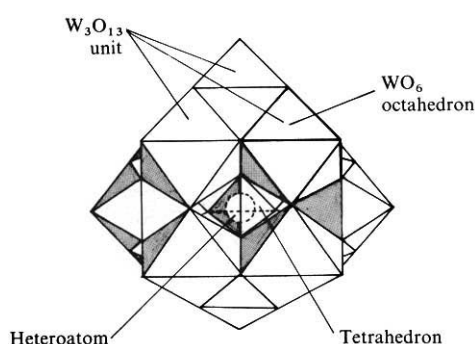


Fig. 3 Schematic structure of heteropolyanion $(\text{XW}_{12}\text{O}_{40})^{m-}$ with the Keggin structure. A heteroatom such as P is placed in the centre of the tetrahedron, whereas carbon in these compounds may occupy the centre of one of the tetrahedral surfaces.

with common edges) are linked by common corners to give a tetrahedral cavity (Fig. 3). A heteroatom such as P at the centre then provides a tetrahedral PO_4 group with four oxygens for each W_3O_{13} unit. Specific bands due to vibration of the XO_4 group can be seen in infrared and Raman spectra of compounds with this structure.

The infrared band around $1,400\text{ cm}^{-1}$ is, however, distinctive for the present compounds and must be assigned to one of the vibrations due to the C—O bonds (Fig. 2). Although the CO_4 group, the orthocarbonate anion, is formally equivalent to PO_4 or SiO_4 , this is an uncommon grouping for carbon which is found only exceptionally in esters such as $\text{C}(\text{OCH}_3)_4$. Moreover, the vibrational frequencies due to CO_4 are unlikely to appear in the vicinity of $1,400\text{ cm}^{-1}$.

It is, therefore, more reasonable to suppose that carbon in these compounds is formed into the familiar CO_3 group. An infrared active band (or bands) due to the ν_3 of this planar group is usually found in the range $1,400$ – $1,500\text{ cm}^{-1}$ as, for example, in calcite⁷. From among the vibrational frequencies observed in the infrared and Raman spectra, those which can be assigned to the fundamentals of CO_3 are shown in Table 1. Other bands below $1,000\text{ cm}^{-1}$ are attributed to the WO_6 groups.

The symmetry (D_{3h}) of CO_3 in an isolated state is usually lowered in both the solid state and in solution by interactions with ligands or surrounding molecules, resulting in modified selection rules and/or the splitting of degenerate bands. The two infrared bands at $1,350$ and $1,400\text{ cm}^{-1}$ for solid I are due to the splitting of ν_3 at sites of C_s symmetry, but the corresponding frequency is not split in solids II and III, indicating D_3 symmetry. The Raman bands in solids I and II at 950 – 960 cm^{-1} , which are due to the totally symmetric vibration (ν_1), are Raman-active only in D_3 symmetry and cannot be detected in the infrared spectra of my samples. The frequencies ν_2 of solids II

and III are seen at 880 – 890 cm^{-1} only in the infrared spectra, in agreement with the selection rule. In the case of C_s symmetry, all four vibrations must be active in both infrared and Raman modes, and these bands are indeed found in the spectra of solid I except for the Raman band ν_3 , which was presumably not detected because the signals are too weak.

These observations are evidence for the existence of a novel heteropolyacid, $\text{H}_4(\text{CW}_{12}\text{O}_{40}) \cdot n\text{H}_2\text{O}$, based on the anion $(\text{CW}_{12}\text{O}_{40})^{4-}$ (or, more properly, $(\text{H}_2\text{CW}_{12}\text{O}_{40})^{2-}$ or $(\text{H}_3\text{CW}_{12}\text{O}_{40})^{-}$) which incorporates the CO_3 group. This may be a kind of carbonato-complex in which carbon occupies the centre of one surface of the tetrahedron in the Keggin structure, to give rise to bonds with three oxygens out of four belonging to each W_3O_{13} ligand.

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Major ion chemistry of the Ganga–Brahmaputra river systems, India

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Rivers are the main pathways by which continental weathering products are transported to the oceans so that the chemistry of river waters and the flux of elements transported by them to the oceans are central to our understanding of the exogenic cycles of elements. There have been several detailed studies of major rivers^{1–5} and we now report the major ion chemistry of another, the Ganga–Brahmaputra. These are two of the large rivers which drain the Indian subcontinent and they transport annually $\sim 10^{15}$ l of water and $\sim 2 \times 10^{15}$ g suspended matter to the Bay of Bengal^{6,7}. On a global scale, these rivers rank fourth in terms of flow, and first in terms of sediment transport^{6,7}. Studies on the continental denudation rates and the budgets of major elements in the ocean, would, therefore, be incomplete without good geochemical data on these rivers. Our studies show that the Ganga–Brahmaputra system transports some 118 million tons of dissolved solids annually to the Bay of Bengal and that their water chemistry is dictated by the weathering of carbonates and contributions from soil salts and/or saline groundwaters.

The Ganga originates in the Gangotri glacier in the Kumaun Himalayas and is known as the 'Bhagirathi' at its source. The Alaknanda is the major tributary of the Bhagirathi, and after their confluence at Devprayag the river acquires the name 'Ganga' (Fig. 1). The major tributaries of the Ganga are the Gomti, Ghaghara and Gandak from the north and the Yamuna and Son from the south. The Chambal, Betwa and Ken are the major tributaries of the Yamuna. Although all these rivers are perennial, the peak flow in all of them occurs during the south-west monsoon, July–September. The Brahmaputra originates in the Chamyungdung glacier in the Tibetan Himalayas. Several tributaries join the main river in the Assam valley, a major one being the Manas. The Ganga and the Brahmaputra merge in Bangladesh, before their outfall into the Bay of Bengal.

The Ganga and its tributaries were sampled during three seasons, March, September and December 1982, representing

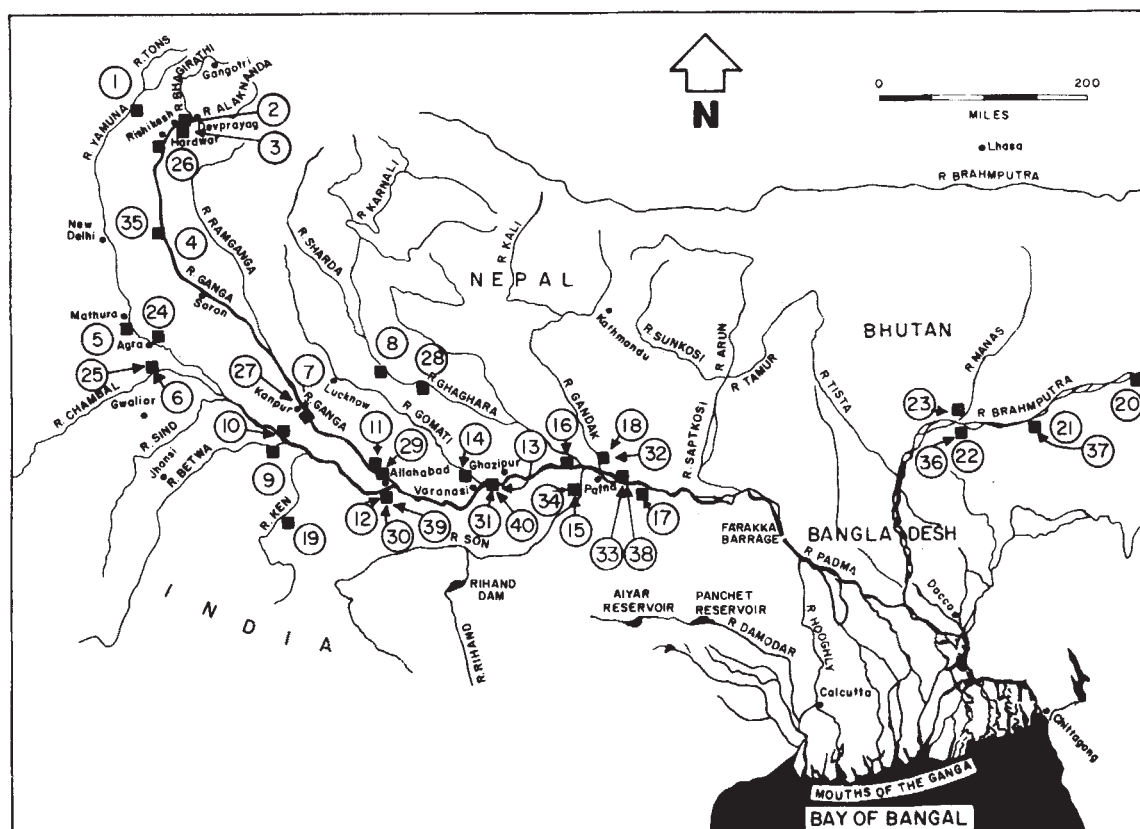


Fig. 1 Sample location map. Numbers correspond to sampling sites in March–April (1–23); September (24–35) and December (36–40) 1982.

Table 1 Major ion composition ($\mu\text{mol l}^{-1}$) of the Ganga and Brahmaputra rivers and their tributaries

Sample code*	Location	Na	K	Mg	Ca	HCO ₃	Cl	SO ₄	SiO ₂	TDS (mg l ⁻¹)
Highland rivers										
26, Ganga	Hardwar	59	38	153	353	894	23	121	109	94
28, Ghaghara	Ayodhya	96	59	232	528	1,601	26	76	114	144
32, Gandak	Hajipur	74	84	259	649	1,860	32	103	143	170
22, Brahmaputra	Goalpara	80	46	148	318	884	29	99	118	92
23, Manas	Goalpara	94	42	181	348	930	26	113	131	99
Lowland rivers										
24, Yamuna	Agra	1,401	130	395	794	1,921	1,172	412	170	287
30, Yamuna	Allahabad	556	57	190	547	1,753	222	67	164	173
25, Chambal	Dholpur	593	53	337	829	2,531	284	76	296	247
34, Son	Ara	240	40	166	434	1,372	71	22	210	129
Ganga main channel										
26, Ganga	Hardwar	59	38	153	353	894	23	121	109	94
35, Ganga	Garhmukteshwar	105	68	182	471	1,159	19	143	127	121
27, Ganga	Kanpur	354	82	242	565	1,829	78	103	157	173
29, Ganga	Allahabad	425	79	201	458	1,570	116	85	121	151
31, Ganga	Varanasi	473	62	188	514	1,707	162	72	147	164
33, Ganga	Patna	280	63	203	547	1,707	87	58	129	156

Measurements were taken during peak flow for all rivers (September 1982) except for the Brahmaputra and Manas, for which measurements were taken during moderate flow (April 1982).

* Numbers correspond to locations in Fig. 1.

respectively lean, peak and moderate flow conditions. Samples from the Brahmaputra and Manas were collected during April and December 1982 (see Fig. 1 for the sampling sites). All the samples were taken from surface waters, collected from the mid-channel of the river either from highway bridges or by going into the rivers using small boats. Details of sampling and their pretreatment are discussed elsewhere⁸. pH, dissolved O₂, conductance and bicarbonate were measured on site. An aliquot of the water sample was filtered within ~6 h of collection through a 0.45- μm Millipore filter using a polycarbonate filter system⁸.

The filtered water was split into two fractions, one of which was preserved unacidified and the other acidified to pH ~2 using HNO₃. The filtered unacidified samples were analysed in the laboratory for major cations and anions using appropriate methods⁸. The major ion composition of the Ganga and its tributaries during their peak flow (samples 24–35; Fig. 1) and that of the Brahmaputra and Manas during their moderate flow (samples 22 and 23; Fig. 1) are given in Table 1. The 'highland rivers' cover the Ganga (up to Hardwar), Ghaghara, Gandak, Brahmaputra and Manas, the 'lowland rivers' the Yamuna (at

Table 2 Average major ion composition ($\mu\text{mol l}^{-1}$) of the Ganga and Brahmaputra rivers and their tributaries

River	Na	K	Mg	Ca	HCO ₃	Cl	SO ₄	SiO ₂	TDS (mg l ⁻¹)
Ganga*	533	83	257	475	1,751	146	112	112	170
Ghaghara	131	61	270	562	1,777	29	82	124	159
Gandak	104	85	285	655	1,886	52	130	147	177
Yamuna†	933	62	290	573	2,183	382	120	157	222
Chambal	827	56	374	792	2,627	337	120	260	262
Betwa	2,377	53	690	502	4,238	426	71	237	388
Ken	701	54	461	550	2,683	233	52	214	241
Son	277	43	191	464	1,507	93	27	214	142
Manas	94	42	181	348	930	26	113	131	99
Brahmaputra	92	48	157	351	957	31	106	130	100
Ganga‡	419	67	269	583	1,966	140	83	132	183
World average§	191	38	166	398	1,019	105	96	207	115

See text for calculations of the average composition.

* At Allahabad, before confluence with the Yamuna; † at Allahabad, before confluence with the Ganga; ‡ at Patna, after confluence of the Ghaghara, Son and Gandak; § from ref. 4.

Allahabad), Chambal and Son. For brevity, the results from other seasons are not given here, but they are used to derive the average chemical composition of these river waters (Table 2).

The major ions in river waters are supplied from the atmosphere and from the weathering of rocks/soils in the drainage basin. The atmospheric contribution consists of two components, the marine cycle salts and the terrestrial inputs. Our study did not include the chemical analysis of rain water and thus it is difficult to obtain a precise estimate of the atmospheric supply of major ions to the river waters during the sampling period. However, the atmospheric contribution of marine cycle salts has been estimated from the available rain water data⁹, assuming that the measured chloride concentration in the highland rivers, during their peak flow, is solely due to the marine contribution⁸. These calculations suggest that, during peak flow, about 30% of sodium in the highland rivers is of marine origin⁸. Of the lowland rivers, the Son has the largest marine component, 45% Cl and 11% Na. The marine contribution of other major ions to these rivers is negligible. The estimated marine cycle salt contribution is an upper limit in that we have assumed that no chloride is supplied to the river water by weathering processes. That contribution can be taken to be small from the observation that the Cl concentration in highland rivers, during their peak flow (23–32 $\mu\text{mol l}^{-1}$, Table 1), is very similar to that in rain water from inland stations (18–41 $\mu\text{mol l}^{-1}$, ref. 8).

In the highland rivers, Ca, Mg and HCO₃ are the most abundant dissolved species in all the seasons sampled. The cations Ca and Mg account for 84–92% of total cations (on an equivalent basis); the (Ca + Mg): (Na + K) ratio ranges between 5.2 and 11.5. HCO₃ constitutes 73–91% of the anions, sulphate accounting for most of the remainder (Tables 1, 2). These abundances favour carbonate weathering as source, which is

consistent with the regional lithology of the rivers, whose upper reaches are composed of Siwalik sediments and the limestones, dolomites and calcareous shales of the Deoban–Tejam zone^{10,11}. The sulphate in the waters may originate from the weathering of gypsum/anhydrite or by the oxidation of pyrite; in the Kumaun Himalayas, gypsum, has been reported as a common accessory mineral¹⁰ and in the Brahmaputra basin, pyritic sediments are present^{4,11}. Furthermore, the low abundance of silica and the high (Ca + Mg): (Na + K) ratios suggest that the contribution of major ions to these waters by silicate weathering has only minor significance.

In the Yamuna (at Allahabad), and the Chambal and Son during peak flow, (Ca + Mg) accounts for 71–81% of the total cations and HCO₃ accounts for 83–92% of the anions (Table 1). As in the highland rivers, the dominance of Ca, Mg and HCO₃ in the major ion composition is attributable to carbonate weathering. The Chambal drains the Vindhyan sandstones, quartzites, siliceous limestones and dolomites, whereas the stretch of the Yamuna between Saharanpur and Allahabad consists of alluvium rich in 'Kankar' carbonates¹². These three rivers were also sampled during the lean flow season. The water chemistry of the Son shows no significant variation with the seasons. In contrast, in the Yamuna and the Chambal, the major ion ratios differ significantly with the flow conditions⁸. During lean flow, the concentrations of Na, Cl and SO₄ increase considerably and account for nearly a third of the major ions, the alkaline and saline soil salts in the drainage basins and/or effluent seepage of saline groundwaters. The other lowland rivers, the Gomti, Betwa and Ken, were sampled only during lean flow (Table 2). The water chemistries of the Son and Ken are similar. During lean flow, the Betwa waters are very saline and are second only to the Yamuna in terms of the abundance of total dissolved salts (TDS).

Table 3 Average annual fluxes (10^9 mol yr^{-1}) of dissolved species through Ganga–Brahmaputra river system

River	Discharge ($10^{12} \text{ l yr}^{-1}$)	Na	K	Mg	Ca	HCO ₃	Cl	SO ₄	SiO ₂	TDS (M ton yr^{-1})
Ganga*	59	31	4.9	15	28	103	8.6	6.6	6.6	10
Yamuna	93	87	5.8	27	53	203	36	11	15	21
Chambal	30	25	1.7	11	24	79	10	3.6	7.8	7.8
Ghaghara	94	12	5.8	25	53	168	2.7	7.8	12	15
Son	32	8.9	1.4	6.1	15	48	3	0.9	6.9	4.5
Gandak	52	5.4	4.4	15	34	98	2.7	6.8	7.7	9.2
Ganga†	364	153	24	98	212	716	51	30	48	67
Brahmaputra‡	510	47	24	80	179	488	16	54	66	51
World rivers§	31,400	6,000	1,200	5,200	12,500		3,300	3,000	6,500	3,600

Discharge data from ref. 18.

* At Allahabad before confluence with Yamuna; † At Patna after confluence with Ghaghara, Son and Gandak; ‡ at Goalpara; § from ref. 4.

The downstream and seasonal variations in the major ion chemistry of the Ganga main channel (Table 1) are controlled by the chemistry of its tributaries and their mixing volumes. The $(\text{Ca} + \text{Mg}) : (\text{Na} + \text{K})$ equivalent ratio decreases downstream, but more markedly during lean flow^{8,14}, when the ratio becomes as low as 1.25 at Varanasi, after the confluence of the Yamuna. The relative increase of $(\text{Na} + \text{K})$ during lean flow is attributable to supply from soil salts in the alluvial plains and/or effluent seepage from saline groundwaters. The TDS of the Ganga main channel, during peak flow, range between 94 and 173 mg l⁻¹ between Hardwar and Patna (Table 1). The maximum TDS concentration, 401 mg l⁻¹, was observed during lean flow at Varanasi. The TDS concentration and range that we observed in the Ganga main channel, during peak flow, is about a factor of three lower than that reported¹⁵ (227–502 mg l⁻¹) during the wet monsoon in 1977.

The average major ion compositions of the rivers sampled are given in Table 2. For the Ganga and its tributaries, the average concentration, $\bar{C}$, was calculated as $\bar{C} = 0.83 C_p + 0.17 C_l$ where C_p and C_l are the concentration of the species during peak and lean flow conditions. The factors 0.83 and 0.17 are based on the monthly discharge data for the Ganga at Farakka¹⁶, which show that 83% of water transport occurs during July–September and the remaining 17% during the rest of the year. The major ion abundances, measured in September and March 1982, are taken to be typical of peak and lean flow conditions. The discharge data for the Brahmaputra at Gauhati¹⁷ show that moderate/peak flow conditions occur during April–October and that 86% of water discharge is during this period. For the Brahmaputra, $\bar{C} = 0.86 C$ (April) + 0.14 C (December). For the Betwa, Ken and Manas, the measurements were taken during one season only. Although these results are presented as their average composition (Table 2), they are not used for flux calculations.

A comparison of the average chemical composition and the TDS content of the Ganga (at Patna) and the Brahmaputra (at Goalpara) shows that the Ganga waters are nearly twice as saline as those of Brahmaputra (Table 2). Although the water flux of the Ganga is ~70% of that of the Brahmaputra, the annual flux of dissolved salts carried by the Ganga (at Patna), 67 million tons, is ~30% more than that of the Brahmaputra at Goalpara. The Ganga transports nearly three times more Na and Cl than the Brahmaputra. The Na and Cl to the Ganga comes mainly from its tributaries, the Yamuna and the Chambal (Tables 1, 3).

On a global scale, the average TDS content of the Ganga and its tributaries is higher than that of the global mean river water composition (Table 2). The average TDS content of the Ganga (at Patna) is very similar to that of the Chiang Jiang of China⁴ and about five times more than that of the Zaire and Amazon⁴. The Ganga accounts for ~2.5% of the global flux of Na to the oceans (Table 3). The Ganga (at Patna) and the Brahmaputra (at Goalpara) together supply ~118 million tons yr⁻¹ of dissolved solids to the Bay of Bengal. This accounts for ~3% of the global supply of dissolved salts to the oceans, which is nearly the same as their contribution to the global water discharge.

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Complexity of human T-cell antigen receptor β -chain constant- and variable-region genes

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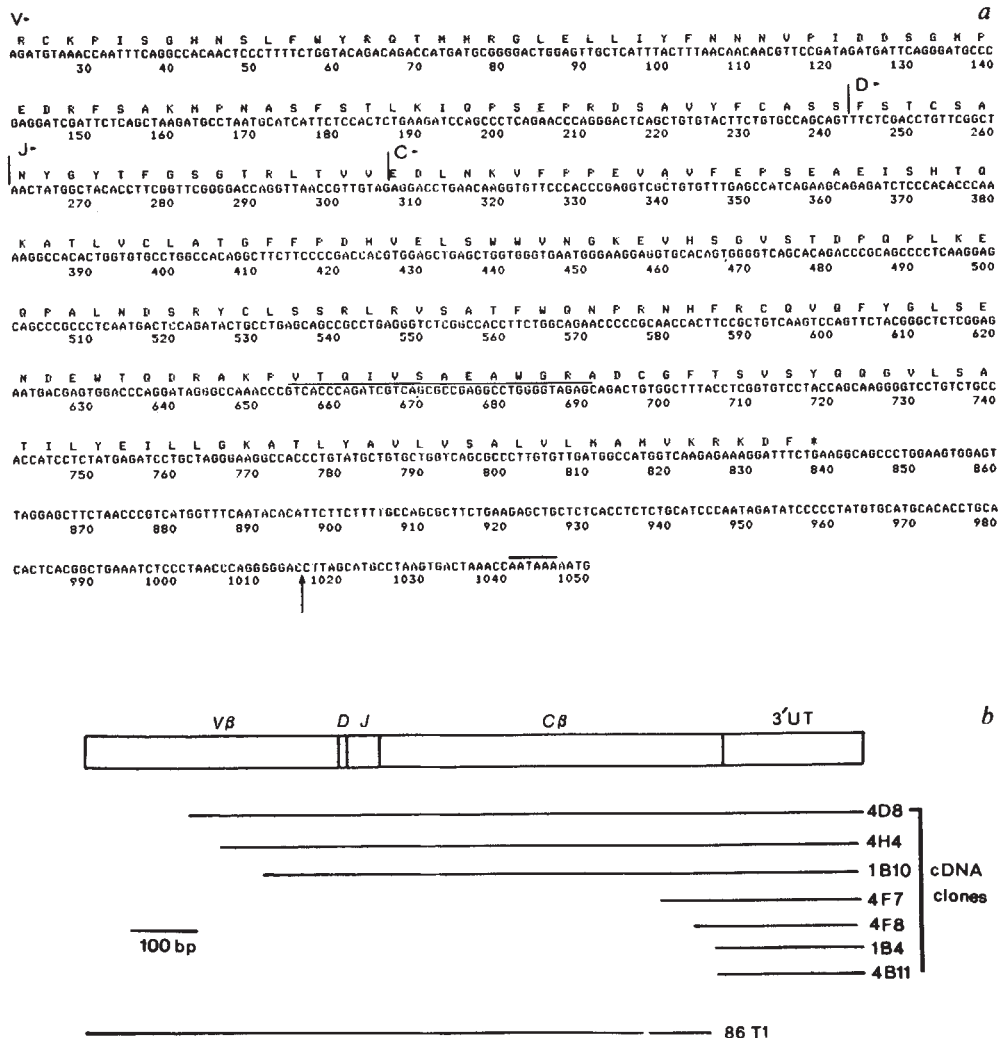
Immune systems of vertebrates function via two types of effector cells, B and T cells, which are capable of antigen-specific recognition. The immunoglobulins, which serve as antigen receptors on B cells, have been well characterized with respect to gene structure¹, unlike the T-cell receptors. Recently, cDNA clones thought to correspond to the β -chain locus^{2–4} of the human and mouse T-cell receptor have been described. The presumptive β -chain clones detect gene rearrangement specifically in T-cell DNA and show homology with immunoglobulin light chains. The similarity of the T-cell β -chain gene system to the immunoglobulin genes has been further demonstrated by the recent observation of variable- and constant-region gene segments as well as joining segments and putative diversity segments^{5,6}. We report here the characterization of cDNA and genomic clones encoding human T-cell receptor β -chain genes. There are two constant-region genes ($C_{\beta 1}$ and $C_{\beta 2}$), each capable of rearrangement and expression as RNA. The gene arrangement, analogous to that of mouse β -chain genes^{6–8}, shows strong evolutionary conservation of the dual C_{β} gene system in these two species.

The JM cell line, positive for the surface markers T1, T3, T4, T6 and T8 (ref. 9 and unpublished data), was derived from a human acute lymphoblastic T-cell leukaemia patient¹⁰. A cDNA library of 500 clones was prepared from JM RNA after removing sequences present in the cDNA that were homologous to B-cell mRNA (ref. 3; Fig. 1 legend). Clones encoding T-cell receptor β -chain sequences were isolated from this library in two related experiments. First, we screened a subset of 188 JM cDNA clones with the isolated insert from the mouse β -chain clone 86T1 (ref. 3) and found four hybridizing colonies (pJMT1B10, 4D8, 4F7 and 4H4). Independently, we had been characterizing individual clones in the library by using them as probes on Northern blots containing T- and B-cell RNA. When the isolated insert from one of these T cell-specific clones (clone pJMT1B4) identified in this way was used to screen the same subset of 188 clones, it hybridized to the same four clones identified by the mouse β -chain probe, as well as to another two clones (pJMT4B11 and 4F8). (Clones 1B4, 4B11 and 4F8 fail to hybridize with 86T1 because they do not overlap with it; see Fig. 1.) We conclude that this group of seven clones is probably derived from the β -chain mRNA synthesized in the JM cell line.

The nucleotide sequence of the presumptive β -chain cDNA clones and the derived amino-acid sequence (Fig. 1) show that the clones derive from the homologous human T-cell receptor β -chain, as predicted from their hybridization with the mouse β -chain probe. The 3' portion of the sequence encodes the constant (C)-region segment (C_{β}) extending from nucleotide 306 to the termination codon at residue 837, and continues as a presumptive 3'-untranslated sequence. Other sections of the sequence correspond to a joining (J) sequence (nucleotides

Fig. 1 Nucleotide sequence of the β -chain cDNA clones isolated from JM RNA. **a**, DNA sequence: the positions of *V*, *D*, *J* and *C* regions are indicated, with the predicted amino acid sequence. The first codon (specifying arginine) is equivalent to codon 41 of the previously published YT35 sequence² and the complete sequence is identical with YT35 except for an extra T residue at JM position 884 in the 3'-untranslated sequence. The vertical arrow shows the position of the last residue of the YT35 sequence. The sequence AATAAA, characteristic of poly(A) addition sites¹¹, is overlined. The sequence in the coding region indicated by a line corresponds to the region used for orientation in Fig. 3. **b**, Organization of the β -chain mRNA, with the extent of the various cDNA clones covering this mRNA. DNA sequencing of the β -chain cDNA clones was carried out in M13 vectors by shotgun cloning and sequencing of *Bgl*II, *Taq*I, *Sau*3A and *Eco*RI plus *Bam*HI digests of pJMT-4D8 using dideoxy chain termination procedures¹⁵⁻¹⁹.

Methods: cDNA clones were prepared from RNA isolated essentially as described²⁰. Single-stranded cDNA was synthesized on methyl mercuric hydroxide-denatured JM poly(A)⁺ RNA and the template subsequently hydrolysed using recommended conditions²¹. The mixture was passed over a Sepharose CL-4B column in 50 mM NaOH, 1 mM EDTA, and fractions containing cDNA longer than ~200 nucleotides were precipitated with ethanol and transfer RNA carrier and resuspended in H₂O. cDNA (3 μ g) was hybridized with 300 μ g of B-cell RNA (from the Burkitt's lymphoma CA46)²² (estimated to be about a 15-fold molar excess of RNA to cDNA, based on average sizes of 700 and 2,000 nucleotides for cDNA and RNA, respectively, and the % rRNA contaminating the poly(A)⁺ RNA) in 360 μ l of 0.2 M NaPO₄, pH 6.8, 1 mM EDTA, for 16 h at 70 °C (to a R_{0t} actual of 150). The mixture was diluted with 30 vol. of 0.02 M NaPO₄, pH 6.8, bound to hydroxyapatite at room temperature, and single-stranded cDNA then eluted from the resin with 0.12 M NaPO₄, pH 6.8 at 60 °C. After rebinding and re-eluting this material, 10.6% of the total cDNA behaved as if it were single-stranded. Thus, the hybridization procedure should have resulted in approximately a 10-fold enrichment for T cell-specific sequences, assuming a 2% difference in the complexity of T- and B-cell RNA populations²³. After removal of NaPO₄ by gel filtration on Sephadex G-100, the cDNA was made double-stranded, treated with *S*₁ nuclease, size-selected again on Sepharose CL-4B, repaired with the Klenow fragment of DNA polymerase I, and ligated into *Sma*I-cleaved and phosphatase-treated pUC8 (ref. 24) essentially as described elsewhere²¹. Ampicillin-resistant colonies were selected after transformation into competent²⁵ LE392 (ref. 21). The yield was ~10⁶ colonies per μ g double-stranded cDNA. Approximately 5,000 colonies were screened with ³²P-labelled cDNA probes made from total poly(A)⁺ RNA of JM or CA46 cells. About 500 which apparently hybridized to the T-cell and not the B-cell cDNA probe were picked, and these comprise the T cell-enriched library referred to in this paper.



216-305), to a small diversity (*D*) sequence (nucleotides 243-260), and the remainder to a part of a variable (*V*) region segment². The *J* segment present in the JM clones is identical in sequence to the germ-line segment *J* _{β} 1-2 (ref. 5). Furthermore, the entire sequence, including the presumptive *V*, *D* and *J* segments, is identical with a β -chain sequence reported to derive from the human T-cell line MOLT-3 (ref. 2), except for one base. In addition, our sequence extends for another 36 residues at the 3' end, including the sequence AATAAA, probably representing the poly(A) addition signal in this mRNA¹¹.

The identity between the sequences of the β -chain cDNA clone derived from JM cells and a clone derived supposedly from the MOLT-3 cell line² is surprising. JM and MOLT-3 were isolated from different T-cell leukaemias and it is unlikely that identical *V-D-J* joining would have occurred independently in these cell lines. We have thus investigated the expression of *V* _{β} sequences in mRNA from six different human leukaemic cell lines, including JM and MOLT-3 with *V* _{β} and *C* _{β} probes

(Fig. 2). All six T-cell leukaemias express an RNA of ~1.35 kilobases (kb) which hybridizes with the *C* _{β} probe, showing that they all transcribe a *C* _{β} gene. However, only JM RNA hybridized to the *V* _{β} probe derived from the JM cDNA clones. Thus, although MOLT-3 cells express the *C* _{β} gene, they do not express a *V* _{β} segment homologous to the JM sequence. This conclusion is supported by Southern hybridization data. We have shown that three independent sources of JM cellular DNA, as well as J6 (a subclone of the JM cell line) DNA, contain identical *C* _{β} rearrangements, which are different from those present in two independent sources of MOLT-3 DNA. The identity of β -chain cDNA clones from JM (reported here) and another source of MOLT-3 (ref. 2) can therefore be attributed to trivial, not physiologically significant, causes.

The *C* _{β} probe hybridizes to a second, smaller species in some of the T-cell RNA preparations (Fig. 2). The origin of this RNA is unknown, although as the *V* _{β} probe detects only the larger of the two transcripts, the smaller may represent sterile *C* _{β}

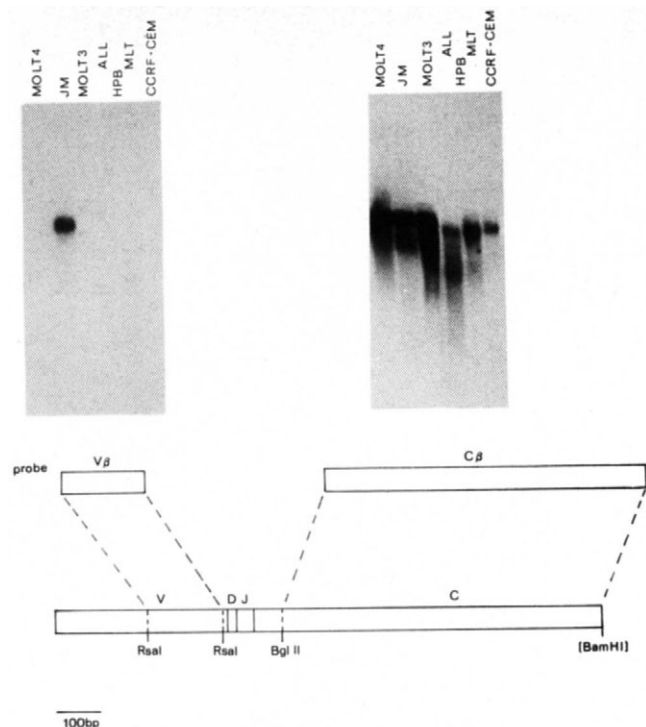


Fig. 2 Northern filter hybridization of V_{β} - and C_{β} -specific probes with RNA prepared from various T-cell leukaemia lines. Duplicate Northern filters were prepared and hybridized essentially as described²⁶ with either 1 μ g poly(A)⁺ RNA (in the JM and MOLT-4 lanes) or 20 μ g total RNA (in the other lanes) fractionated on 1.5% agarose gels. One filter was probed with pUCV_{JM} (V_{β}) and the other with M131B10BB1 (C_{β}). Note that the *Bam*HI site at the 3' end of the diagram of the C_{β} cDNA insert is from the pUC vector polylinker site. V and C region-specific probes were prepared by subcloning from pJMT4D8. The C_{β} probe was an M13 clone extending from the *Bgl*II site, just within the C_{β} sequence (nucleotide 364 of fig. 1) to the *Bam*HI site located in the pUC vector polylinker segment just beyond the 3' end of the insert. The V_{JM} probe was a clone containing the 165-base pair (bp) *Rsa*I fragment (nucleotides 59–224 of Fig. 1), cloned into the *Sma*I site of pUC9. The cell lines MOLT-4 and MOLT-3 (ref. 9), CCRF-CEM²⁷, HPB-ALL and HPB-MLT, are from T-cell leukaemias.

transcripts lacking V segments, analogous to those sometimes made from the immunoglobulin C_{μ} gene in T cells¹².

The genomic arrangement of the C_{β} genes was studied by isolating clones from a human B-cell genomic λ phage library¹³ screened with the mouse β -chain clone, 86T1. Two overlapping clones were obtained, λ B3 and λ B17 (Fig. 3). Hybridization of these clones with a human C_{β} probe indicated the presence of two separate C_{β} genes about 10 kb apart. One gene contains a site for the restriction enzyme *Hind*III (see Fig. 3); as there is no *Hind*III site in the cDNA clone of the JM β -chain, we thought that this site might occur within an intervening sequence of the gene. We therefore attempted to orient this C_{β} gene by nucleotide sequencing from the *Hind*III site. λ B3 was digested with *Hind*III together with *Eco*RI and the resulting fragments cloned into M13mp18. Clones carrying the small *Hind*III–*Eco*RI fragment were sequenced from the *Hind*III site towards the *Eco*RI site (Fig. 3). The 237 nucleotides adjacent to the *Hind*III site were not recognizable as C_{β} gene sequence but after this point, the sequence matches a part of the C_{β} segment found in the JM C_{β} cDNA clone (underlined in the cDNA sequence of Fig. 1). A typical RNA splicing signal sequence at the junction of this region probably represents an intron/exon boundary conserved in evolution; one of the mouse C_{β} genes also contains an intron at exactly the same position^{7,8}. Comparison of the genomic and cDNA sequences indicates that the gene containing the *Hind*III site is transcribed from left to right on the map of Fig. 3. Further, other sequencing and restriction

mapping data show that this C_{β} gene differs from that present in the JM cDNA clones, and that the two C_{β} genes are transcribed in the same direction (A.T. and T.H.R., manuscript in preparation). These genes are designated $C_{\beta}1$ and $C_{\beta}2$ (see Fig. 3) by analogy with the two C_{β} genes in mouse^{7,8}.

The existence of two C_{β} genes was confirmed by Southern filter hybridization experiments. For example, digestion of DNA from the B-cell leukaemia D-PLL with *Eco*RI revealed two hybridizing fragments (11.5 and 4 kb; Fig. 4). (D-PLL has been shown to contain a germ-line C_{β} gene pattern; unpublished results.) We know from the λ clones that the $C_{\beta}2$ gene occurs in the 4-kb *Eco*RI fragment, therefore the 11.5-kb germ-line *Eco*RI fragment must contain the $C_{\beta}1$ gene. We also failed to detect any closely related C_{β} genes other than those present in the genomic λ clones.

Several different patterns of DNA rearrangement can occur in T-cell leukaemias (Fig. 4). For example, MOLT-3 DNA has both $C_{\beta}2$ genes rearranged. When MOLT-3 genomic DNA was digested with *Eco*RI and hybridized with the C_{β} probe, we found a single hybridizing band of 4 kb corresponding to $C_{\beta}2$. There was no evidence of any rearranged band or of the 11.5-kb $C_{\beta}1$ germ-line band. Therefore, MOLT-3 apparently does not contain any remnant of the $C_{\beta}1$ gene; thus it has probably been deleted from the relevant chromosomes (assuming no chromosome loss), presumably by V -gene rearrangement to the $C_{\beta}2$ gene. This interpretation was confirmed by *Sac*I digestion of MOLT-3 DNA, again revealing only the unrearranged $C_{\beta}2$ gene fragment (5.4 kb). Digestion of MOLT-3 DNA with *Hind*III, however, does show rearrangement within the $C_{\beta}2$ gene region (Fig. 4). The $C_{\beta}2$ gene overlaps two *Hind*III fragments (8.5 and 7 kb long) in unrearranged DNA such as D-PLL (Fig. 4). The 7-kb fragment, which contains the 3' end of the $C_{\beta}2$ gene, is present in MOLT-3 DNA, whereas the 8.5-kb band, containing the 5' end of the $C_{\beta}2$ gene, is absent (Fig. 4) and two new fragments (26 and 19 kb) are present, indicating rearrangement of the $C_{\beta}2$ gene on both chromosomes in MOLT-3.

An example of a single C_{β} gene rearrangement is shown by JM DNA, which shows 11.5- and 4-kb germ-line C_{β} fragments, and a new, rearranged fragment of 9.5 kb after *Eco*RI digestion. As the $C_{\beta}1$ gene is transcribed in JM, this new band must represent the rearranged $C_{\beta}1$ allele. The other $C_{\beta}1$ allele is apparently not rearranged. If gene deletion is required for $C_{\beta}2$ joining, the existence of this unrearranged $C_{\beta}1$ allele implies that the second chromosome in JM has the germ-line configuration of C_{β} genes. It is unlikely that the different fragment sizes observed are due to polymorphism in the human population as studies of six non-T-cell DNAs failed to detect restriction site polymorphisms with the enzymes used here.

In all T-cell DNAs examined so far (JM and MOLT-3 DNAs plus DNA from 10 fresh leukaemic samples), we have never observed an altered *Eco*RI or *Sac*I pattern around the $C_{\beta}2$ gene. Rearrangements at $C_{\beta}2$ can be detected, however, using *Hind*III. This implies, by analogy with the mouse C_{β} gene system^{7,8}, that each human C_{β} gene has its own joining (J) cluster. The J cluster associated with $C_{\beta}2$ lies outside both the 4-kb *Eco*RI and 5.4-kb *Sac*I fragments but within the 8.5-kb *Hind*III fragment.

Significant variability exists at the level of V_{β} sequences, as the JM V_{β} sequence hybridizes only to RNA from this cell line (Fig. 2). To investigate this further, we studied the hybridization pattern of the V_{β} probe with genomic DNA. Southern filter hybridizations using our V_{β} probe (pUCV_{JM}) reveal six major hybridizing bands in JM DNA (Fig. 4). MOLT-3 DNA contains the same bands with differing hybridization intensities, as well as three faintly hybridizing elements at ~3 kb which are absent from the JM DNA. (In similar experiments, a V_{β} probe related to that used here hybridized to four bands in genomic DNA⁵.) The complexity of the V_{β} hybridization pattern is comparable with that seen in human immunoglobulin V -gene probes¹⁴, suggesting a significant germ-line contribution to V_{β} diversity.

Our results show that the human T-cell receptor β -chain locus consists of two C genes, separated by ~10 kb of DNA, and

Fig. 3 Organization of the human C_β locus. **a**, The top line shows a restriction map of the C_β locus derived from the two indicated overlapping λ phage clones ($\lambda\beta 3$ and $\lambda\beta 17$). The most 5' *EcoRI* site (dotted line) is absent from the phage clones and was determined by genomic hybridizations. The location of the $C_\beta 1$ gene was determined by digestion of $\lambda\beta 3$ with *EcoRI* and *Bgl*II or with *Hind*III and *Bgl*II (*Bgl*II cuts within the $C_\beta 1$ coding sequence). λ phage containing genomic sequences were isolated from a library made by cloning Raji DNA partially digested with *Mbo*I (ref. 13) into $\lambda 2004$ (ref. 28), initially using the mouse clone 86T1 (ref. 3) as the probe. Raji cells derive from a Burkitt's lymphoma patient²⁹. As the phage clones were derived from a B-cell library, we cannot be certain at present that the 5' end of clone $\lambda\beta 3$ is equivalent to germ-line DNA. **b**, The nucleotide sequence used to orient the C_β genes was obtained by subcloning into M13mp18 and partially sequencing the 1-kb *Hind*III-*Eco*RI fragment. The sequenced part is indicated in Fig. 1.

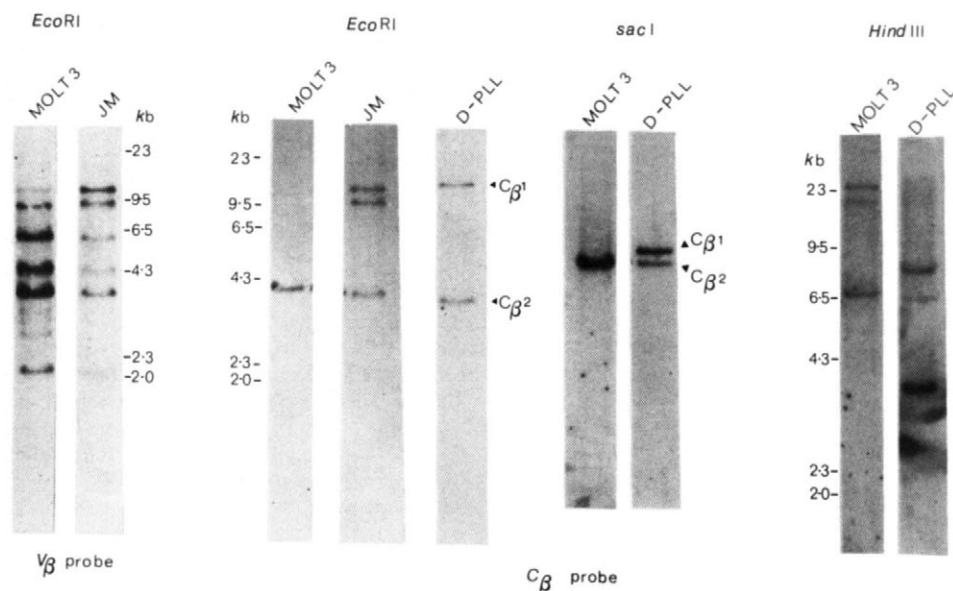
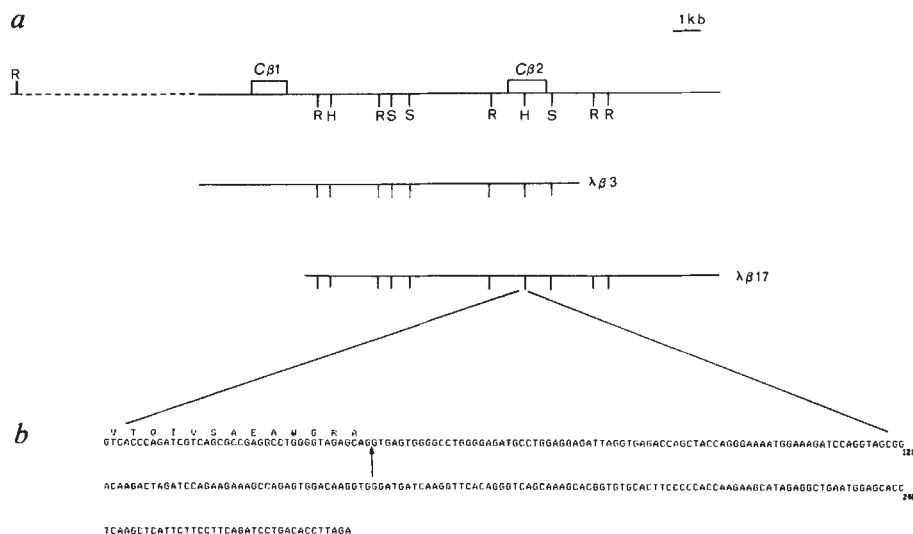


Fig. 4 Southern filter hybridizations of genomic DNA with V_{β} and C_{β} probes. Genomic DNA ($10 \mu\text{g}$) was digested with restriction enzymes, followed by electrophoresis in 0.8% agarose gels and transfer to nitrocellulose filters³⁰. The filters were subsequently hybridized to nick-translated³¹ DNA probes (specific activity 1.4×10^8 c.p.m. μg^{-1}) in $6 \times$ SSC, 0.1% SDS, 5% dextran sulphate, $50 \mu\text{g ml}^{-1}$ sonicated, denatured salmon sperm DNA plus 0.2% Ficoll, 0.2% bovine serum albumin and 0.2% polyvinylpyrrolidone³². After hybridization for approximately 18 h at 65°C , the filters were washed in $0.1 \times$ SSC, 0.1% SDS (5×10 min at 65°C) and autoradiographed at -70°C with pre-fogged film³³. Sizes were determined by co-electrophoresis of λ phage DNA cut with *Hind*III. D-PLL is leukaemic DNA from a patient with prolymphocytic leukaemia and possesses the germline C_{β} gene profile (unpublished data).

multiple V_{β} segments. Each C_{β} gene is functional, as $C_{\beta}1$ is expressed in the JM cell line, whereas $C_{\beta}2$ is rearranged and presumably expressed in MOLT-3.

There are several similarities between the T-cell receptor β -gene locus and the immunoglobulin gene families: for example, the occurrence of deletions associated with gene rearrangements and the existence of multiple inherited V segments (as well as D and J segments) giving considerable potential for diversity. The inherited diversity of human V_β genes is reasonably high as judged by filter hybridization and analysis of V-gene expression in a limited panel of T-cell lines. Perhaps the existence of separate J_β clusters associated with each C_β gene^{7,8} is another strategy required to increase diversity. The similarity of the human and murine β -chain gene organization suggests that the occurrence of two tandem and highly homologous C_β genes may have functional significance. It will be important to establish the extent of germ-line V_β and D_β diversity, to investigate V-gene expression in antigen-specific T-cell clones, and particularly to study the contribution of the α -chain genes.

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Note added in proof: We have now received a sample of MOLT-3 DNA from Dr T. Mak. The pattern of C_β gene rearrangement in this DNA is identical to that of our various JM DNA samples (by *EcoRI* digestion). We also find that our JM DNA has a rearrangement of one immunoglobulin J_H allele (T.H.R., manuscript in preparation); the MOLT-3 DNA from Dr T. Mak has an identical J_H rearrangement whereas our own MOLT-3 DNA samples have a germ-line J_H pattern.

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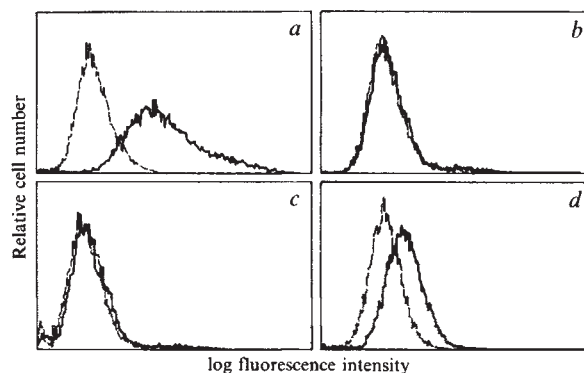


Fig. 1 FACS histograms of cells stained with monoclonal antibody 7.16.4. Histograms representing cell populations stained with monoclonal antibody and FITC-conjugated rabbit anti-mouse immunoglobulin are represented by solid lines (—) while control populations stained with FITC-conjugated rabbit anti-mouse immunoglobulin alone are represented by broken lines (---). *a*, *neu* gene transfectant cell line, B104-1-1. *b*, Normal NIH 3T3 cells. *c*, *Ha-ras* transfectant cell line, XHT-1-1a. *d*, Activated *neu* gene donor rat neuroblastoma, B104.

Methods: Tumour cell lines were grown in tissue culture dishes in Dulbecco's modified Eagle's supplemented with 10% fetal calf serum (FCS), 1% penicillin-streptomycin-Fungizone and 1% gentamycin. Cells were removed from dishes with buffered EDTA (Versene; Gibco) and washed twice in FACS medium (Hank's balanced salt solution (HBSS; Gibco) supplemented with 2% FCS, 0.1% sodium azide and 10 mM HEPES); 1×10^6 cells in 0.1 ml FACS medium were incubated with 0.1 ml of hybridoma culture supernatant for 1 h at 4°C. Cells were washed twice with FACS medium, and incubated with 0.1 ml FITC-conjugated rabbit anti-mouse immunoglobulin (Miles) diluted 1:50 in FACS medium or 1 h at 4°C. Cells were then washed twice in FACS medium and fixed in 2% paraformaldehyde-phosphate-buffered saline (PBS). Samples were run on a Coulter Epics V fluorescence-activated cell sorter. We routinely analysed 10,000 cells per sample. To control for nonspecific binding of the FITC-conjugated rabbit anti-mouse antibody to tumour cells, parallel samples were run which were stained with this reagent alone.

Monoclonal antibodies identify a cell-surface antigen associated with an activated cellular oncogene

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A variety of antigens have been identified on the surface of the malignant cell. However, identical antigens are often found on non-malignant cells of the same or different histological origin, or of a different stage of embryonic development¹⁻⁵. Many of these tumour-associated antigens appear to be only incidentally expressed on neoplastic cells. Clearly, it would be of great interest to identify cell-surface antigens whose expression is associated specifically with the transformed state and linked directly with the mechanisms responsible for transformation. The detection of activated cellular oncogenes in human and animal cancer cells by the technique of DNA transfection has allowed the isolation of genetic elements which are thought to have a critical role in malignancy^{6,7}. Here, in an effort to identify cell-surface antigens associated with the neoplastic process, we have generated hybridomas which secrete monoclonal antibodies that react specifically with cell-surface determinants found on NIH 3T3 cells transformed by transfection with a group of rat neuroblastoma oncogenes. These antibodies bind to and immunoprecipitate a phosphoprotein of relative molecular mass 185,000 (185 K) from a DNA donor rat neuroblastoma and 13 independent rat neuroblastoma DNA transfectants. There was no antibody reactivity with normal NIH 3T3 cells or with NIH 3T3 cells transformed by various other agents.

We have described previously a group of oncogenes found in the genomes of several ethylnitrosourea-induced rat neuroblastomas; these oncogenes induced neoplastic transformation of mouse NIH 3T3 cells when introduced by the technique of DNA transfection⁸. Inoculation of young mice with NIH 3T3 transfectants transformed by rat neuroblastoma DNA resulted in the appearance of fibrosarcomas. Sera from the tumour-bearing mice immunoprecipitated a 185K phosphoprotein (p185) that was present in all mouse cells that had acquired the rat oncogene⁹. p185 was absent from mouse cells that had been transformed by other oncogenes, including the *ras* oncogenes. Moreover, mouse cells transformed by the rat neuroblastoma oncogene lack increased levels or altered versions of the known *ras* oncogenes and their p21 products¹⁰. These data suggest that synthesis of p185 is specifically induced by the rat neuroblastoma oncogene, and that this oncogene is distinct in its behaviour from other known oncogenes. We refer to members of this family of oncogenes by the term '*neu*'.

Preliminary experiments suggested that *neu*-transfected NIH 3T3 cells displayed transformation-specific antigens on their surface. These experiments used polyclonal sera raised in mice injected with *neu*-transfected NIH 3T3 cells emulsified in Freund's adjuvant. Immunofluorescence tests showed reactivity of antiserum with intact transfected cells which was not affected by extensive pre-absorption on normal NIH 3T3 cells (data not shown). Based on these results, we used indirect immunofluorescence as a screening technique in our efforts to develop monoclonal antibodies reactive with cell-surface determinants on *neu*-transfected cells. Briefly, C₃H/HeJ mice were repeatedly immunized with NIH 3T3 transfectants transformed by the *neu* oncogene (cell line B104-1-1), emulsified in Freund's adjuvant.

Table 1 Expression of p185 on murine tumour cell lines

Cell line	Description	Immunofluorescence
NIH 3T3	Contact-inhibited mouse fibroblast	-
B104-2	1 ⁰ Transfectant of B104 rat neuroblastoma DNA	+
B104-1-1	2 ⁰ Transfectant of B104 DNA	++++
B104-1-2	2 ⁰ Transfectant of B104 DNA	+
B103-1	1 ⁰ Transfectant of B103 rat neuroblastoma DNA	+++
B103-5	1 ⁰ Transfectant of B103 DNA	++
B103-1-1	2 ⁰ Transfectant of B103 DNA	++
B103-1-2	2 ⁰ Transfectant of B103 DNA	+
B50-1	1 ⁰ Transfectant of B50 rat neuroblastoma DNA	+
B50-6a-2	2 ⁰ Transfectant of B50 DNA	++++
B50-6a-3	2 ⁰ Transfectant of B50 DNA	++
B82-1	1 ⁰ Transfectant of B82 rat neuroblastoma DNA	+
B82-3	1 ⁰ Transfectant of B82 DNA	+++
B82-3-2	2 ⁰ Transfectant of B82 DNA	+
XHT-1-1a	2 ⁰ Transfectant of v-Ha-ras proviral DNA	-
RSV 3T3	RSV-transformed NIH 3T3	-
MSV 3T3	Moloney sarcoma virus-transformed NIH 3T3	-
HaSV 3T3	Harvey sarcoma virus-transformed NIH 3T3	-
PyD2	Polyoma virus-transformed mouse fibroblast	-
PyBR	Polyoma virus-transformed mouse fibroblast	-
SVD2	SV40-transformed mouse fibroblast	-
S1509a	3-Methylcholanthrene-transformed mouse fibroblast	-
YAC	Mo MuLV-induced lymphoma	-
SP2	Mineral oil-induced plasmacytoma	-

Cell lines were prepared for FACS analysis on a Coulter Epics V Cell Sorter using saturating amounts of monoclonal antibody 7.16.4 as described in Fig. 1 legend. Immunofluorescence was quantitated by subtracting the median fluorescence channel of populations stained with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin alone (negative control) from the median fluorescence channel of populations stained with monoclonal antibody 7.16.4 followed by FITC-conjugated rabbit anti-mouse (positive staining). The resulting net fluorescence was expressed according to the following scale: -, less than two channels; +, 5-10 channels; ++, 11-15 channels; +++, 16-20 channels; +++++, >20 channels. 1⁰ and 2⁰ indicate primary or secondary transfectants created by passing the oncogene through one or two sequential cycles of DNA transfection in NIH 3T3 mouse fibroblasts. MoMuLV, Moloney murine leukaemia virus.

Spleens from immune mice were fused with the aminopterin-sensitive NS-1 myeloma line, and hybridomas were selected in hypoxanthine-aminopterin-thymidine media. Culture supernatants from growing hybridomas were initially screened for the presence of antibody capable of binding B104-1-1 cells by indirect immunofluorescence using fluorescence activated cell sorting (FACS). Positive supernatants were then tested for specificity by determining whether they contained antibody capable of binding normal NIH 3T3 cells, or NIH 3T3 cells transformed by transfection with Harvey sarcoma virus proviral DNA (cell line XHT-1-1a).

To date, we have screened ~3,000 hybridomas and have identified five that produce monoclonal antibodies capable of specifically binding B104-1-1 cells. One of these hybridomas, that produces an antibody termed 7.16.4, will be described in detail. It secretes immunoglobulin of the IgG2a isotype and has maintained production of specific antibody through three cycles of limiting dilution cloning and two cycles of soft agar cloning. Figure 1 shows FACS histograms representing the binding of monoclonal antibody 7.16.4 to several different cell lines. FACS

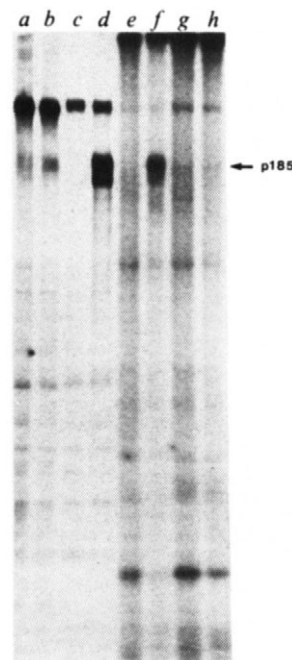


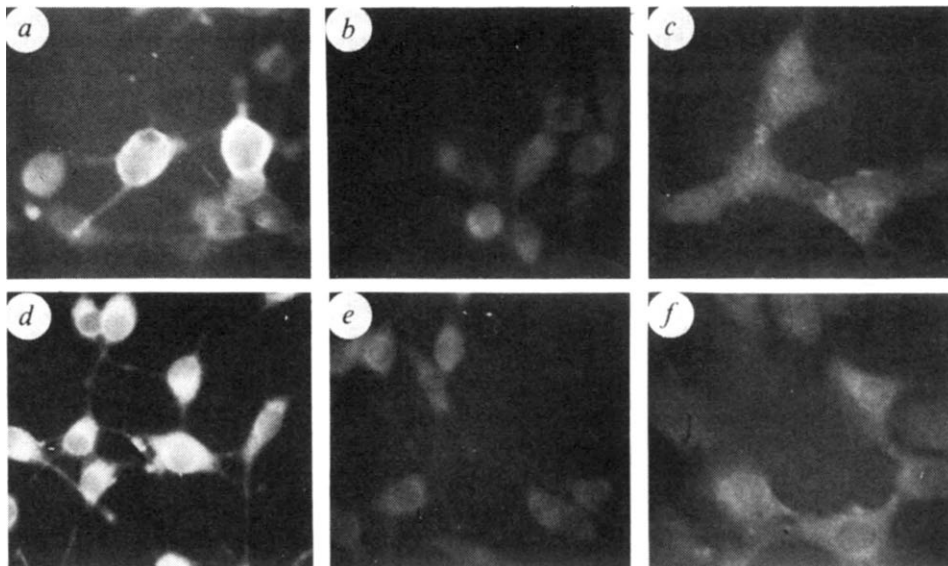
Fig. 2 Immunoprecipitation of metabolically labelled p185 by monoclonal antibody 7.16.4. *a-d*, ³²S-cysteine-labelled cell lysates: *a*, NIH 3T3 lysate precipitated with normal mouse serum (NMS); *b*, NIH 3T3 lysate precipitated with monoclonal antibody 7.16.4; *c*, *neu* gene transfectant B104-1-1 lysate precipitated with NMS; *d*, B104-1-1 lysate precipitated with 7.16.4. *e-h*, ³²P-labelled cell lysates: *e*, B104-1-1 lysate precipitated with NMS; *f*, B104-1-1 lysate precipitated with 7.16.4; *g*, NIH 3T3 lysate precipitated with NMS; *h*, NIH 3T3 lysate precipitated with 7.16.4.

Methods: For labelling with ³⁵S-cysteine, 10⁶ cells were seeded in 100-mm culture dishes and labelled for 18 h in 2 ml minimal essential medium (MEM) containing 0.1 the usual amount of cysteine, 2% dialysed FCS and 500 μCi ³⁵S-cysteine (77 Ci mmol⁻¹; NEN). For labelling with ³²P, 3 × 10⁵ cells were seeded in 60-mm tissue culture dishes and incubated for 18 h in 0.8 ml phosphate-free Dulbecco-Vogt modified Eagle's medium containing 4% FCS and 0.4 mCi ³²P (carrier-free; NEN). Cells were lysed in phosphate-buffered RIPA buffer containing 1 mM ATP, 2 mM EDTA and 20 mM sodium fluoride, and immunoprecipitates were prepared and washed as described elsewhere²². One-third of each lysate was incubated with 1 μl of normal mouse serum or 60 × concentrated 7.16.4 culture supernatant at 4 °C for 60 min. Sheep anti-mouse immunoglobulin (1 μl; Cappel) was added to each sample, and incubation continued for 30 min. Immune complexes were pelleted using fixed Protein A-bearing *Staphylococcus aureus* and washed. Samples were analysed by SDS-polyacrylamide gel electrophoresis in 7.5% acrylamide-0.17% bisacrylamide gels²². The gels were treated for fluorography and exposed to preflashed Kodak X-Omat AR film for 10 days.

can quantitate antibody binding to particular cell populations by immunofluorescence, displaying more brightly stained populations (those which bind more antibody) to the right of less brightly stained populations. Figure 1 shows strong binding of the antibody to cell line B104-1-1 (*a*), and significant, although weaker, binding to the DNA donor, the rat neuroblastoma cell line, B104 (*d*). Antibody 7.16.4 binds neither to nontransformed NIH 3T3 cells (Fig. 1*b*) nor to NIH 3T3 cells transformed by a Harvey *ras* (Ha-*ras*) oncogene (Fig. 1*c*). Thus, this antibody is directed against neither a normal NIH 3T3 cell-surface antigen nor an antigen associated in a nonspecific fashion with either proliferation or neoplastic transformation of murine cells. Antibody 7.16.4 appears to recognize a cell-surface antigenic determinant specifically expressed by cells containing the *neu* oncogene.

Immunofluorescent staining for FACS analysis was performed on cells that were initially >90% viable as determined by Trypan blue exclusion. However, we considered it formally possible that cell death during antibody staining might allow antibody to enter dead cells and cause cell fluorescence due to

Fig. 3 Indirect immunofluorescent staining of B104-1-1 and NIH 3T3 cells. *a-c*, Paraformaldehyde-fixed cells: *a*, *neu* gene transfectant B104-1-1 cells stained with 7.16.4 plus FITC-conjugated rabbit anti-mouse antibody; *b*, B104-1-1 cells stained with FITC-conjugated rabbit anti-mouse antibody alone; *c*, NIH 3T3 cells stained with 7.16.4 plus FITC-conjugated rabbit anti-mouse antibody. *d-f*, Methanol-fixed cells: *d*, B104-1-1 cells stained with 7.16.4 plus FITC-conjugated rabbit anti-mouse antibody; *e*, B104-1-1 cells stained with FITC-conjugated rabbit anti-mouse antibody alone; *f*, NIH 3T3 cells stained with 7.16.4 plus FITC-conjugated rabbit anti-mouse antibody. NIH 3T3 cells fixed with either paraformaldehyde or methanol and stained with FITC-conjugated rabbit anti-mouse antibody alone showed staining comparable to that in *c* and *d* (data not shown).



Methods: Cells were grown on glass coverslips for 48 h, then the coverslips were washed in PBS and fixed with either 2% paraformaldehyde-PBS or absolute methanol for 10 min at room temperature. Coverslips were then washed in FACS medium and incubated with 10-fold concentrated 7.16.4 culture supernatant or FACS medium (for controls) for 30 min at 4°C. They were then washed in FACS medium and stained with FITC-conjugated rabbit anti-mouse immunoglobulin (diluted 1:50; Miles) for an additional 30 min. The coverslips were extensively washed in FACS medium, washed in PBS, and antibody was fixed *in situ* by a 10-min incubation in 2% paraformaldehyde-PBS. Coverslips were mounted on glass slides and photographed using 20-s exposures to Kodak Tri-X pan film with a Leitz fluorescence microscope at $\times 400$ magnification.

the binding of intracellular rather than cell-surface antigenic determinants. We excluded this possibility by performing FACS analysis on live cells stained with fluorescent antibodies in the presence of propidium iodide. Propidium iodide is excluded from live cells, but enters dead cells, where it intercalates with nucleic acids and emits a red fluorescence when excited by the FACS 488-nm argon laser¹¹. By setting the cell sorter to exclude cells displaying such red fluorescence, we confirmed that monoclonal antibody 7.16.4 reacts with a determinant on the surface of intact B104-1-1 cells (data not shown).

The specificity of the antibody was further examined by immunoprecipitation of metabolically labelled cell lysates. Figure 2 demonstrates that monoclonal antibody 7.16.4 immunoprecipitates a phosphoprotein with a relative molecular mass of ~ 185 K (p185) from the *neu*⁺ NIH 3T3 transfectant B104-1-1 (Fig. 2*d, f*). Several other faint bands are also visible in both normal mouse serum immunoprecipitates and monoclonal antibody 7.16.4 immunoprecipitates of B104-1-1 cell lysates (Fig. 2*c-f*). A faint band of about the same size is seen in NIH 3T3 lysates immunoprecipitated with either normal mouse serum or monoclonal antibody (Fig. 2*a, b, g, h*); because this band is slightly different in molecular weight from p185, and is also precipitated by normal mouse serum, we conclude that there is no specific precipitation of p185 from normal NIH 3T3 cells by monoclonal antibody 7.16.4. The 185K phosphoprotein identified by antibody 7.16.4 appears to be identical to the *neu* oncogene-associated p185 identified previously using polyclonal sera (data not shown).

FACS analysis, using monoclonal antibody 7.16.4, of a large number of murine cells transformed by a variety of agents has revealed that p185 is expressed only in cells transformed by *neu* oncogenes (Table 1). By this test, NIH 3T3 cells transformed by several retroviruses containing known oncogenes, including *src*, *mos* and *ras*, lack p185. Similarly, mouse fibroblast lines transformed by the papovaviruses simian virus 40 (SV40) and polyoma virus, as well as the chemical carcinogen 3-methylcholanthrene, are also not stained by monoclonal antibody 7.16.4. Murine tumours of non-fibroblast histological origin, such as the SP₂ plasmacytoma and the YAC T-cell lymphoma, also fail to bind anti-p185 monoclonal antibody. In contrast, all of 13 NIH 3T3 transfectants transformed by *neu* oncogenes contain significant amounts of cell-surface p185, as determined by antibody binding. It is interesting that the level of p185

expression varies somewhat between transfectants; cell lines that show the highest expression of p185 as determined by immunofluorescence also contain the most p185 as determined by quantitative immunoprecipitation (data not shown). Although a very high level of p185 is seen in some transfectants, such a high level does not seem to be necessary for oncogenic transformation of NIH 3T3 cells. The absolute correlation between the expression of cell-surface p185 and the presence of rat *neu* oncogenes in murine transfectants suggests, but does not prove, that p185 is a *neu* oncogene product.

Some oncogene-encoded proteins have been localized to particular functional regions within the cell: for example, the Rous sarcoma virus (RSV) transforming gene product, pp60^{src}, is found in high concentration adjacent to cellular adhesion plaques¹². In an effort to determine whether such cytological compartmentalization exists for p185, we performed indirect immunofluorescence on non-permeabilized and permeabilized B104-1-1 cells grown on coverslips (Fig. 3). Apparently, p185 is distributed in a diffuse pattern on the extracellular face of the plasma membrane of paraformaldehyde-fixed cells (Fig. 3*a*). In contrast, cells permeabilized by fixation in absolute methanol display diffuse antibody binding throughout the cell, and are particularly well stained in the perinuclear region of the cytoplasm (Fig. 3*d*). It remains to be determined whether these results reflect compartmentalization of p185 precursor proteins undergoing processing on membrane-associated ribosomes or in the Golgi apparatus, or whether they are simply a result of the thicker cross-section in this region of the cell. p185 does not appear to be specifically associated with sites of cellular adhesion or cell-cell contact.

Several proteins have been associated with the various activated oncogenes described in non-virally-induced tumour cells and cell lines¹³⁻¹⁵. However, none of these proteins is similar in size to the 185K antigen associated with the *neu* oncogene and none has been identified as a cell-surface protein. Furthermore, none of the proteins known to be encoded by retroviral oncogenes has physical properties identical to those of p185, although the gene products of *v-fms* and *v-erb-B* and their cellular homologues may also have portions of the respective molecules exposed on the cell surface¹⁶⁻¹⁸. The data presented here and in ref. 10 strongly suggest that p185 is a *neu* oncogene product.

The cell-surface localization of p185 may offer insight into a

potential mechanism of oncogene action. p185 has many characteristics similar to those of the receptors for epidermal growth factor (EGF)¹⁹ and platelet-derived growth factor (PDGF)²⁰, including approximate molecular weight, plasma membrane localization and phosphorylation. p185 is also a glycoprotein (D.F.S., unpublished results), as are the EGF and PDGF receptors. Waterfield and colleagues have presented persuasive evidence that the viral *erb-B* oncogene represents a truncated EGF receptor gene which lacks the extracellular EGF-binding domain but nonetheless delivers to the cell a mitogenic signal which results in neoplastic transformation²¹. The *neu* gene product p185 may also be an altered growth factor receptor which transforms cells by a mechanism similar to that of *v-erb-B*. Its localization to the cell surface and association with oncogene

activation suggest that p185 may prove useful as a potential target for directing monoclonal antibodies against tumours containing activated *neu* oncogenes; we are presently investigating this possibility.

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Therapy with monoclonal antibodies by elimination of T-cell subsets *in vivo*

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A major aim in immunology has been to understand how the immune system evokes characteristic responses to infection, foreign tissue grafts and tumours. The current view of immunoregulation is based mainly on studies of lymphocyte subsets, either *in vitro*¹⁻³ or by adoptive transfer to irradiated recipients⁴. Many reagents are available for defining T-cell subsets⁵⁻⁸, but only recently have there been helper T-cell-specific antibodies^{9,10} against the mouse equivalent of the Leu3/T4 (man) and W3/25 (rat) antigens. It is clear that monoclonal antibodies¹¹ will eventually replace anti-lymphocyte globulin for immunosuppression in organ grafting^{12,13}, but although there has been some clinical success¹⁴⁻¹⁶, most monoclonal reagents cause only transient reductions in their target cells *in vivo*¹⁷⁻²⁰. This uncertainty in the potency of monoclonal antibodies has led some workers to consider them as targeting agents for such highly cytotoxic drugs as ricin A (ref. 21). We show here that unmodified monoclonal antibodies can be extremely effective at depleting cells *in vivo* and can be used for the selective manipulation of different aspects of the immune response.

The observation that two different rat IgG2b anti-mouse Thy-1 antibodies were potently immunosuppressive when administered *in vivo*, compared with a range of other monoclonal antibodies of the same specificity but different isotypes^{22,23}, suggested that the antibody isotype might be an important factor for serotherapy. We therefore prepared²⁴ rat monoclonal antibodies to several mouse T-cell antigens using the Y3/Ag1.2.3 myeloma²⁵ as fusion partner and compared these for their ability to suppress the *in vivo* antibody response to sheep red blood cells (Fig. 1). Adult thymectomized mice were used so that new T cells would not be produced after monoclonal antibody depletion, and immunosuppression could then be measured in the absence of any short-term effects of the antibody. Only the IgG2b antibodies recognizing an antigen expressed on the helper T cells (Thy-1, Lyt-1 and L3/T4) were effective at eliminating the antibody response. Six rat anti-Thy-1 monoclonal antibodies of other isotypes (IgM, IgG2a and IgG2c) and one IgG2a

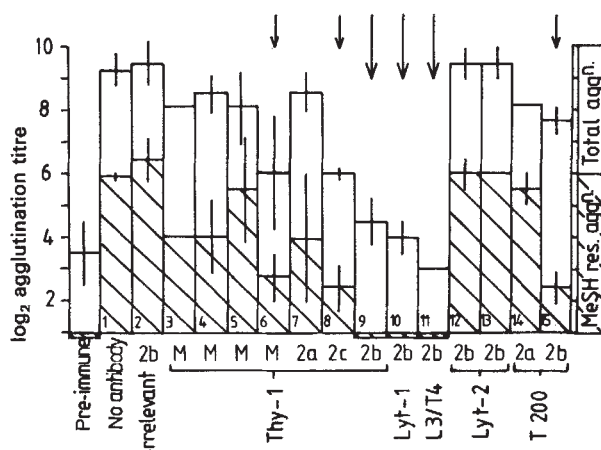


Fig. 1 Suppression of the antibody response to SRBCs. Male CBA mice were thymectomized at 5-6 weeks of age and randomly selected 3 weeks later for monoclonal antibody administration, given as two intravenous injections, 1 week apart, of 0.2 ml of monoclonal ascitic fluid [from (LOU × DA)_{F1} rats], equivalent to ~0.5 mg of active antibody in the following groups: 1, no antibody ($n = 10$); 2, irrelevant IgG2b ($n = 2$, YTH 89.1 anti-human glycoporphin A; G. Hale, personal communication); 3-6, four different IgM anti-mouse Thy-1²³ (YTS 27.3, $n = 2$; YTS 109.8, $n = 2$; YTS 198.3, $n = 2$; YTS 148.3, $n = 4$); 7-9, IgG2a (YTS 111.4, $n = 2$), IgG2c (YBM 29.2, $n = 4$), IgG2b (YTS 154.7, $n = 4$) anti-mouse Thy-1²³; 10, IgG2b anti-mouse Lyt-1 ($n = 4$; YTS 121.5 which cross-inhibits 53-7.313 (ref. 7 and our unpublished observation); 11, IgG2b anti-mouse L3/T4 [$n = 4$; YTS 191.1 which is present on only the Lyt-2 negative peripheral T cells (Table 1), 85-90% of thymocytes and <2% of bone marrow cells, reacts with a protein of relative molecular mass 55,000 as determined by 'Western blots' (ref. 45 and our unpublished observations) and is therefore similar to GK1.5 (ref. 10); 12, 13, two IgG2b anti-Lyt-2 [YTS 169.4, $n = 6$; YTS 172.3, $n = 6$, which cross-inhibit 53-6.72 (ref. 7 and our unpublished observation); 14, 15, IgG2a (YBM 42.2 (ref. 46), $n = 2$) and IgG2b (YW 62.3, $n = 2$, which cross-inhibits YBM 42.2, our unpublished observation) anti-T200 ('leukocyte common antigen'⁴⁷). Animals were given 0.2 ml of 10% SRBCs intraperitoneally 3 weeks after monoclonal antibody (except for preimmune group, $n = 6$). These were bled from the tail vein after 10 days and the serum agglutination (aggⁿ) titre determined with SRBCs alone (total IgM and IgG response) or with SRBCs in the presence of 0.1 M 2-mercaptoethanol (MeSH-resistant IgG response). Substantial immunosuppression is indicated by the arrows.

anti-T200 were far less effective than the IgG2b reagents of the same specificities.

The observed immunosuppression could be accounted for by depletion of helper or total T cells (Table 1). At various times from 1 week to 3 months after the original injection of monoclonal antibody, groups of treated, or control thymectomized, mice were compared for the expression of various T-cell markers using the cytofluorograph. All of the rat IgG2b antibodies gave substantial and long-term depletion (5 weeks post-antibody in Table 1) of their target T cells. Residual rat immunoglobulin could not be detected and an anti-globulin response was observed by day 10 (in all but YTS 191.1-treated mice, results not shown). This means that suppression was unlikely to be due to blocking of function, temporary changes in the pattern of lymphocyte circulation or 'antigenic competition'²⁶, which could contribute to the short-term effects described in other systems^{23,27}, such as that of LeGros *et al.*²⁸.

Selective manipulation of the major T-cell subsets could be readily achieved with the IgG2b monoclonal anti-L3/T4 or -Lyt-2 antibodies (Table 1). For example, if we consider the YTS 169.4 (anti-Lyt-2) treated group, the Lyt-2-positive cells were reduced to 0.2%, which represents a specific depletion of at least 96% compared with thymectomized controls. Elimination of cells was also effective in the lymph-nodes, with 14.5% Lyt-2-positive T cells in controls compared with 1.2% in treated mice (results not shown). The loss of Thy-1, L3/T4 and Lyt-1 in these treated mice was consistent with a reduction in only the Lyt-2-positive T cells. Over a 12-month observation period, there was no observable change in Lyt-2 expression. This demonstrates that any remaining Lyt-2-positive T cells were unable to repopulate the animal and that, in these conditions, the mature Lyt-2-negative phenotype was stable and irreversible, in contrast to some suggestions of phenotypic switching²⁹. Similar long-term depletions were observed in the YTS 191.1 (anti-L3/T4) treated mice, although we have not followed these animals beyond 6 months post-injection. It is possible that further injections of antibodies are required to maintain depletion during a very strong antigenic challenge, where small numbers of residual T cells might be induced to proliferate.

Immunological responses in T-cell subset-depleted mice were those expected considering the surface phenotype of the remaining T cells. Mice depleted of L3/T4-positive cells did not make an antibody response to sheep cells (Fig. 1) or the injected rat immunoglobulin (results not shown), and were also unable to develop delayed hypersensitivity (HSV-DH, Table 1) to herpes simplex virus (recently implicated as an important effector mechanism in immunity to such viruses³⁰ and in allograft rejection³¹). Mice depleted of either subset (L3/T4 or Lyt-2) were unable to produce cytotoxic T cells to allogeneic targets *in vitro* (Table 1, Fig. 2). This is because the generation of cytotoxic T cells depends on the presence of both helper (Lyt-2⁻) and cytotoxic precursor (Lyt-2⁺) cells³²; simple mixing of the cells from the L3/T4- and Lyt-2-depleted animals at the start of the culture period reconstituted the response (Fig. 2). Therefore, animals can be independently depleted of the 'helper' or 'cytotoxic' T-cell lineages by administering monoclonal antibodies to L3/T4 and Lyt-2. These animals have remained functionally depleted for at least 6 months after monoclonal antibody injection. Previously, this could only be achieved, albeit inefficiently, by using populations of T cells depleted of Lyt-1⁺ or Lyt-2⁺ subsets *in vitro* to reconstitute lethally irradiated, thymectomized mice⁴. Such animals may have a contribution from radio-resistant T cells³³, and complications associated with irradiation are avoided by the *in vivo* antibody treatment. The major advantage of using subset-specific monoclonal antibodies directly in animal models is that it should be possible to apply the rules learned in, for example controlling allograft rejection, to therapy in man.

It is still not entirely clear which T cells are responsible for the rejection of skin grafts in mice^{31,34,35}, partly perhaps because of some confusion over Lyt-1 expression³³. We used CBA mice depleted of either L3/T4- or Lyt-2-positive T cells to determine the cells required to reject fully mismatched (BALB/c) or H-2-matched (minor antigens mismatched, B10.BR) skin grafts (Fig. 3). In both cases, Lyt-2 depletion did not delay rejection, while L3/T4 depletion led to greatly increased survival of the grafts. These results were confirmed using normal CBA mice (with a thymus) given a 14-day course of antibody to cover the initial

Table 1 Depletion of T-cell subsets *in vivo* by serotherapy with monoclonal antibodies

Antibody treatment	% Positive spleen cells					% Specific depletion	HSV-DH ($\times 10^{-2}$ mm)	Allo-CTL (% control)
	Thy-1 (154.7)	Lyt-1 (121.5)	Lyt-2 (169.4)	L3/T4 (191.1)	L3/T4: Lyt-2 ratio			
YTS 154.7	1.6(-)	2.3(+)	0.6(-)	ND	ND	90	10.4 $\pm$ 1.5	ND
YTS 121.5	2.5	2.7(-)	0.7(-)	ND	ND	82	5.5 $\pm$ 1.0	21
YTS 169.4	10.3	11.0	0.2(-)	11.3	57	96	13.0 $\pm$ 1.8	2
YTS 191.1	8.8	4.7	5.0	1.5	0.3	86	4.5 $\pm$ 2.0	0
None (ATX)	15.7	14.6	5.1	10.8	2.1	0	15.4 $\pm$ 2.3	100
Normal	33.5	35.0	11.4*	24.3*	2.1	ND	ND	ND

Male CBA mice were thymectomized at 5-6 weeks of age, randomly selected for injection of monoclonal antibodies and tested 3 weeks later for their responses to sheep red blood cells (SRBCs) as described in Fig. 1 legend, HSV-specific delayed hypersensitivity (DH) as described below or generation of allo-CTL as described in Fig. 2. Two mice from each of the SRBC-primed group were selected at random, 5 weeks after monoclonal antibody injection, and the spleen (and mesenteric lymph nodes, data not shown) was taken for analysis of T-cell markers. Red cells were lysed by NH_4Cl treatment and the remaining lymphocytes incubated for 1 h at 4°C in antibody supernatants at 100 $\mu\text{g ml}^{-1}$ of medium containing 5% heat-inactivated normal rabbit serum to block Fc binding, and 0.1% NaN_3 . After washing, bound monoclonal antibody was detected by incubation with fluorescein isothiocyanate (FITC)-labelled mouse monoclonal antibodies to rat immunoglobulin light chains (MAR 18.5)⁴² and rat IgG2b (NORIG 7.16)³⁶. Cells were washed in medium containing 50 $\mu\text{g ml}^{-1}$ propidium iodide to stain dead cells, and resuspended in phenol red free medium containing 0.5% bovine serum albumin and 0.1% NaN_3 . Samples were analysed using the H50 Cytofluorograph with 2150 computer (Ortho Diagnostic) at 1,000 cells s^{-1} . Computer gating was used on forward and 90° light scatters, and on red fluorescence (propidium iodide) such that histograms of green (fluorescein) fluorescence were obtained for live, monomeric cells. Numbers show % positive cells (above fluorescence channel 40 on a linear scale of 1,000 channels: controls with the FITC-labelled anti-globulins alone gave background of 0.1-0.3% positive) and are means of the results obtained from two mice in each group analysed individually. % Specific depletion is the % depletion of cells with the phenotype of injected antibody specificity as compared with ATX controls. Also shown for comparison are results for two normal (not thymectomized) CBA mice. HSV-DH: For the induction of virus-specific delayed hypersensitivity, 10⁵ plaque-forming units (PFU) HSV-1 TK⁻ (strain CL101 deficient in thymidine kinase) was injected into the ear pinna, as previously described⁴³. Seven days later the mice were challenged with 5 $\times$ 10⁵ PFU HSV-1 TK⁻ in the contralateral pinna and the DH response was measured 24 h later as the increase in ear thickness⁴⁴. The results are expressed as the arithmetic mean $\pm$ s.d. of 5 mice per group. CBA mice receiving only the challenge inoculum, as a control for nonspecific inflammation, gave 3.8 $\pm$ 1.2 $\times 10^{-2}$ mm. Allo-CTL: % control cytotoxic T lymphocytes calculated from the effector:target ratio required to give 30% specific lysis (Fig. 2). Plus or minus in parentheses indicates respectively a $\sim$ 10% increase or a 25-50% decrease in mean fluorescence of positive cells. ND, not determined. Underlining emphasizes the depleted population.

* Lyt-2 + L3/T4 gave 38.7% positive.

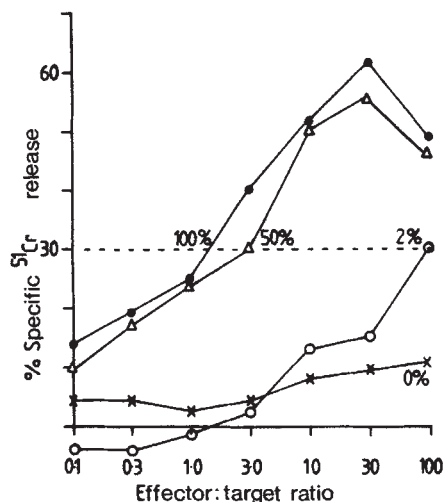


Fig. 2 L3/T4- and Lyt-2-negative mice are independently depleted of the helper and cytotoxic T-cell lineages. ●, Normal thymectomized (ATX) mice; ×, YTS 191.1 (anti-L3/T4) treated ATX mice; ○, YTS 169.4 (anti-Lyt-2) treated ATX mice; Δ, 50:50 mix of cells at start of culture from YTS 191.1 and YTS 169.4 ATX mice. **Methods:** Spleens were taken from two normal, two YTS 191.1 (anti-L3/T4), and two YTS 169.4 (anti-Lyt-2) treated, adult thymectomized male CBA mice, 3 weeks after monoclonal antibody administration. Responder cells from individual mice were incubated at both 2×10^6 and 5×10^6 cells ml^{-1} with 10^6 irradiated (2,500 rad) BALB/c stimulator spleen cells in 1-ml Linbro cultures, in Iscove's modified Dulbecco's medium containing 5% fetal calf serum (FCS) at 37°C for 6 days with constant rocking. Effector cells were collected and pooled from the mixed lymphocyte cultures for each animal and titrated (in triplicate) in 100- μl microtitre wells with 10^4 BALB/c spleen cells that had been previously stimulated with concanavalin A for 2 days before labelling with ^{51}Cr -labelled sodium chromate. The supernatants were removed for γ counting after 4 h at 37°C . Cytotoxic activity was measured as the % specific ^{51}Cr release, calculated as:

$$\% \text{ Specific release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

where the spontaneous release was from labelled targets alone, and the total release was from detergent-treated targets. Effector:target ratios were calculated on the basis of the number of cells added at the start of the mixed lymphocyte culture. Each point is the mean of two experiments. Approximate % of control activity as shown was calculated from the effector to target ratio required to give 30% specific release.

period of grafting with fully allogeneic (BALB/c) skin (mean graft survival in days: no antibody, 11 ± 2 ; anti-Lyt-2, 12 ± 2 ; anti-L3/T4, 25 ± 3 ; anti-Thy-1, 25 ± 3). Immunoperoxidase staining³⁶ of spleen sections taken on the day after rejection confirmed that the target cells were still relatively depleted. These results suggest that T cells with the phenotype Thy-1^+ , L3/T4^+ , Lyt-2^- are the major promoters of primary graft rejection in mice. However, Lyt-2-positive cells may have a secondary role, particularly in fully mismatched grafts, where removal of all T cells was more effective than removing just the L3/T4 cells (Fig. 3) and anti-Lyt-2 injection from day 7 of grafting in previously untreated mice did lead to delayed rejection (mean survival 18 ± 3 days compared with 13 ± 2 days in untreated controls, not shown). This confirms and extends the results obtained by adoptive transfer into lethally irradiated, thymectomized rats where the W3/25⁺ helper T cells were sufficient to cause graft rejection³⁷.

The above results demonstrate that selected rat IgG2b monoclonal antibodies can be very effective *in vivo*. These would also seem to be the best candidates for human therapy³⁸, because rat IgG2b is the only subclass known both to fix human complement^{39,40} and to activate antibody-dependent cell-mediated

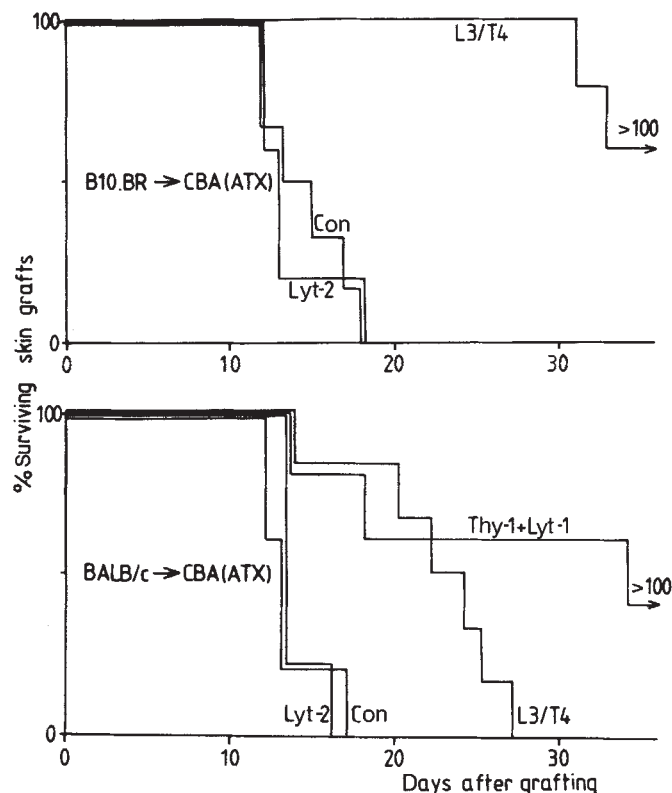


Fig. 3 Suppression of skin allograft rejection with monoclonal antibodies. Per cent surviving grafts are shown for the following groups: Con, controls with no antibody treatment; Lyt-2, YTS 169.4 (anti-Lyt-2) treated group; L3/T4, YTS 191.1 (anti-L3/T4) treated group; Thy-1 + Lyt-1, YTS 154.7 + YTS 121.5-treated group. Groups of 4–10 thymectomized CBA mice were given 2×0.2 ml of rat IgG2b monoclonal antibodies intravenously as described in Fig. 1 legend. BALB/c differing in H-2 and minor histocompatibility antigens or B10.BR (minor histocompatibility differences) tail skin was grafted to the lateral thoracic wall 4 weeks and 8 weeks after antibody treatment respectively, and covered with Vaseline-impregnated gauze and plaster bandage. The dressings were removed on day 10 and the grafts inspected every day for rejection.

cytotoxicity *in vitro*⁴¹, the latter even at very low antibody concentrations. Although most of the above experiments used large (~ 0.5 mg) amounts of antibody, YTS 169.4 is able to deplete Lyt-2 cells, and YTS 191.1 can suppress antibody responses using a total of 1–10 μg per thymectomized mouse.

Using specific monoclonal antibodies to manipulate the immune response, there are at least two ways in which fundamental aspects of immunoregulation can be investigated. First, subset-depleted mice can be used to determine the T cells involved in different aspects of bacterial or viral infections. Such animals are also a source of subset-depleted cells for *in vitro* or adoptive transfer work, with the advantage that no antibody or complement need be present at the time of the experiments. Second, T-cell subsets can be manipulated at any time during a response to foreign antigen or in autoimmune diseases, in order to probe the events which regulate the immune system as a whole. Elimination of cells *in vivo* is also a requirement for the treatment of neoplastic disease, and the above reagents are being used to develop a model for the serotherapy of leukaemias. The use of monoclonal antibodies as therapeutic agents in animal models should allow us to extend strategies for serotherapy which are directly applicable to man.

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Note added in proof: We have recently shown that Lyt-2^+ cells may have a major role in graft rejection when the animals have been previously primed to the graft antigens.

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Dynorphin(1-13) improves survival in cats with focal cerebral ischaemia

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Since the discovery of opiate receptors in the central nervous system (CNS)¹⁻³, it has become apparent that endogenous opiate ligands are involved in CNS function. Most attention has focused on their role in modulating pain, but they have also been implicated in various physiological functions and in disease states. We are concerned with evidence that endogenous opioid peptides may also contribute to the neurological deficits arising from cerebral ischaemia^{4,5}. Dynorphin, which is widely distributed in the brain and pituitary^{6,7}, has been reported to produce unusual motor and behavioural effects⁸⁻¹¹ and may act as a regulatory neuropeptide, not as a classical opiate agonist or antagonist¹²⁻¹⁵. We have therefore administered to cats in which the right middle cerebral artery had been occluded both dynorphin (1-13) and analogue and control materials. We find that dynorphin (1-13) prolongs survival.

Forty-two adult male cats (3-5 kg) assigned randomly to one of four groups were anaesthetized with halothane and subjected transorbital occlusion of the right middle cerebral artery¹⁶. A subcutaneous pocket was created for later placement of an

Table 1 The 18-point scale used for assessing neurological function in cats examined 6 h after middle cerebral artery occlusion

Neurological response	Score
Motor function	
Cat walks with normal gait—no neurological deficit	6
Cat walks with abnormal gait, has mild hemiparesis	5
Cat barely walks with moderate hemiparesis	4
Cat unable to walk with moderate hemiparesis	3
Cat unable to walk with severe hemiparesis	2
Cat unable to walk with hemiplegia	1
Sensory function	
Cat responds appropriately to tactile and noxious stimuli	5
Cat responds appropriately to noxious limb stimuli only	4
Inappropriate response to noxious limb stimulation only	3
Reflex response to noxious limb stimulation only	2
No response to noxious limb stimulation	1
Level of consciousness	
Awake and alert	5
Awake and alert with lack of spontaneous movements	4
Drowsy, responds only to noxious stimuli	3
Stuporous, minimal response to noxious stimuli	2
Comatose	1
Pupillary response	
Unilaterally reactive to light	2
Unilaterally unreactive to light	1

osmotic pump through a small midline lumbar incision, and the cat allowed to awaken. Six hours after occlusion, sensory and motor function, consciousness and pupillary reaction to light were graded on an 18-point scale (Table 1) by two persons who did not know to which group the cat had been assigned. The cats then received an intraperitoneal injection (2 ml) of one of the following solutions: (1) sterile normal saline; (2) Leu-enkephalin, 750 µg kg⁻¹ (1.35 µmol kg⁻¹) in saline; (3) dynorphin(1-13), 2 mg kg⁻¹ (1.25 µmol kg⁻¹) in saline; or (4) dynorphin(3-13), 2 mg kg⁻¹ (1.43 µmol kg⁻¹) in saline. Neurological assessment was repeated 20 min after injection.

Under ketamine sedation, an osmotic pump (Alzet 2MLI, Alza Corporation) was implanted in the subcutaneous pocket to deliver saline at 10 µl h⁻¹, Leu-enkephalin at 20 µg h⁻¹ (36 nmol h⁻¹), dynorphin (1-13) at 50 µg h⁻¹ (31 nmol h⁻¹), or dynorphin (3-13) at 50 µg h⁻¹ (36 nmol h⁻¹). The pumps were coded so that each cat received the drug with which it had been injected earlier.

The cats were housed in metabolic cages at constant temperature and humidity and were examined frequently. Penicillin G, 1 MU per day, was administered intramuscularly; lactated Ringer's solution was injected subcutaneously to provide adequate daily fluid maintenance until the cat began to eat and drink. So long as the cats were alive, their neurological condition was assessed daily by the battery of tests shown in Table 1; 7 days after occlusion all the cats were killed. The brain was removed and a coronal section made at the optic chiasm. The slices were incubated in a 2% solution of 2,3,5-triphenyl-tetrazolium chloride (TTC) for 25 min^{18,19}. Colour slides were made of the stained slices, and the affected hemisphere and infarcted area were traced from projected images of the slide. Using a digitizing pad (BIT PAD ONE, Summagraphics, Fairfield, Connecticut) and a planimetry program on a PDP-11/23 computer, infarct size was calculated as the percentage of infarcted tissue relative to the entire hemisphere.

Neurological scores before and 20 min after acute injection of the peptides were compared by non-parametric analysis of variance (Kruskal-Wallis) followed by *post hoc* individual comparison, and the infarct sizes were compared by one-way analysis of variance. Kaplan-Meier survival curves were constructed and Gehan's statistics used to compare the survival between the groups.

Four additional cats underwent detailed studies of systemic changes associated with acute subcutaneous administration of dynorphin (1-13). After middle cerebral artery occlusion, blood pressure, heart rate, respiration and core temperature were monitored. The radiolabelled microsphere technique of Heyman *et al.*²⁰ was used to determine cardiac output and cerebral blood flow before and after subcutaneous administration of dynorphin (1-13).

Table 2 Survival after middle cerebral artery occlusion in cats

	<24 h	24-48 h	1 week
Saline	0/12 (0%)	0/12 (0%)	0/12 (0%)
Dynorphin (1-13)	7/10 (70%)	6/10 (60%)	6/10 (60%)*
Dynorphin (3-13)	2/10 (20%)	0/10 (0%)	0/10 (0%)
Leu-enkephalin	2/10 (20%)	0/10 (0%)	0/10 (0%)

* $P < 0.001$ compared with control at 1 week.

All cats in the control group died within 48 h of middle cerebral artery occlusion, whereas 60% of the cats that received dynorphin (1-13) survived for 1 week with minimal neurological deficits (Table 2). The small improvements in survival with dynorphin (3-13) and Leu-enkephalin were not statistically significant. There was no statistically significant effect on neurological function or infarct size in any group. Dynorphin (1-13) did not alter blood pressure, heart rate, cardiac output or regional cerebral blood flow.

The observation that dynorphin (1-13) does not influence systemic variables or regional cerebral blood flow suggests that its effects are mediated directly in the CNS. The ineffectiveness of dynorphin (3-13), which lacks the opioid properties of dynorphin (1-13), suggests that the capacity of the latter to enhance survival is mediated through opiate receptors.

Despite reports of the effect of peripherally administered peptides in the CNS²¹, the mode of action of dynorphin (1-13) in this study is unclear. Many peptides, such as the enkephalins, thyrotropin-releasing hormone and somatostatin, penetrate the blood-brain barrier poorly²², but it has also been suggested that even peptides with low brain uptake can enter the brain in appreciable quantities if high plasma concentrations are maintained for long enough²³. Continuous infusion of dynorphin (1-13) by subcutaneously implanted osmotic pump may have achieved this goal.

Our results provide further evidence for the involvement of endogenous opioid peptides in the pathophysiology of cerebral ischaemia. The protective effect of dynorphin (1-13) against death from focal cerebral ischaemia demonstrated here warrants further investigation and suggests possible clinical use of dynorphin (1-13) and its related analogues for human stroke.

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Male sexual differentiation in mice lacking H-Y antigen

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The sexual phenotype of an adult mammal depends on whether the fetal gonad has differentiated as a testis or as an ovary. Because individuals of XY or XXY sex chromosome constitution develop as males, while XX and XO individuals develop as females, the presence of a Y chromosome seems normally to be required for testis differentiation and its absence to be necessary for differentiation of an ovary. The nature of the hypothetical Y-dependent substance responsible for masculinization of the indifferent gonad has been a matter for debate. A male-specific transplantation antigen, H-Y, has been known for many years^{1,2}, and more recently a serologically detected antigen, also male-specific, has been reported³. Those who believe that the two are antigenically distinct^{4,5} refer to the latter as SDM (serologically detected male) antigen, but many refer to both as H-Y antigen⁶⁻⁸. The hypothesis⁹ that H-Y is itself the Y-dependent testis inducer, although supported by little or no direct evidence^{4,10}, is economical and hence attractive. H-Y antigen is frequently stated to be the substance responsible for primary sex determination (for example, see ref. 11). We report here that H-Y is absent from certain mice that develop testes and are of indisputably male phenotype, hence this transplantation antigen is unlikely to be responsible for testis determination.

The mice examined form part of a stock segregating for *Sex-reversed* (*Sxr*)^{12,13}. *Sxr* causes XX embryos to develop as phenotypically normal though sterile males; XY carrier males are now known to possess an extra copy, transposed to a position distal to the X-Y pairing region^{14,15}, of a region of the Y chromosome located near the centromere (Fig. 1). Because of its new location, the transposed region is transferred to the X chromosome by crossing-over during male meiosis¹⁶⁻¹⁸ and, as the resulting X/X^{Sxr} mice develop as males, the region is presumed to include the testis-determining factor *Tdy* (Fig. 1). However, the masculinizing activity can be partly or wholly suppressed if the *Sxr*-carrying X chromosome is partnered by the differentially active T(X; 16)16H X-autosome translocation (T16H)^{19,20}. T16H/X^{Sxr} mice may develop as females, males or hermaphrodites; we presume that they are mosaics of female-determining cells in which the X-inactivation process has spread into the translocated *Sxr* region, and male-determining cells in which *Sxr* is still expressed. The sexual phenotype of an individual would then depend on the proportions of the two types of cell within the gonad primordium. *Sxr*-carrying females constructed in this way are fully fertile.

X/X^{Sxr} males (and T16H/X^{Sxr} males, which in the absence of banded karyotyping cannot be distinguished from X/X^{Sxr} males) are positive for H-Y transplantation antigen^{21,22}, showing that the translocated region contains the gene controlling H-Y,

whether or not this is identical with *Tdy*. We have shown recently that T16H/X^{Sxr} females also are positive for H-Y, whether tested by an *in vitro* cytotoxic T-cell assay or by the proliferative responses of H-Y-specific T-cell clones²³. If these *Sxr*-carrying females are indeed mosaics, containing a cell population in which the *Sxr* sequences are expressed, this result is not surprising. It is interesting that the presence of H-Y antigen in no way interferes with female reproduction.

However, one *Sxr*-carrying female out of 11 tested²³ consistently proved to be negative for H-Y antigen (female 39 in Table 1). This result could reflect one extreme of a mosaic distribution, in which virtually all cells have expression of the *Sxr* region suppressed by X-inactivation; such a situation would be a property of the individual and would not be inherited. Alternatively, it could reflect an alteration in the *Sxr* region, such that the masculinizing activity is retained but the antigenicity associated with the gene for H-Y is no longer expressed. If

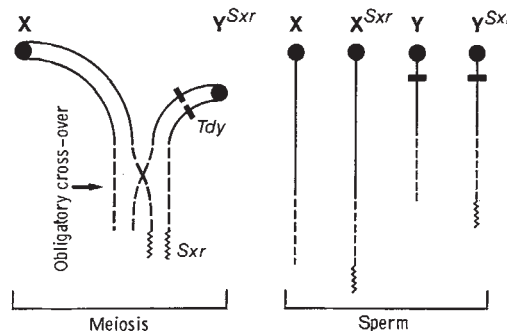


Fig. 1 Inheritance of *Sxr*. *Sxr* carrier males produce four different types of sperm, and hence progeny, because of the single obligatory cross-over between X and Y chromatids during male meiosis. The broken lines indicate pairing regions in the X and Y chromosomes.

Table 1 H-Y typing of mice shown in Fig. 2

Expt	Sex	Source of stimulating target cells (serial no.)	Genotype	Cytotoxicity				Proliferation (c.p.m. $\times 10^{-2}$)				Summary of typing	
				Anti-H-2 ^k	Anti-H-2 ^b	Anti-H-Y ^k	Anti-H-Y ^b	C-3/A-4 (D ^k +H-Y)	Clone (antigen specificity): 10-2-12 (D ^b +H-Y)	2-1-1 (I-A ^b +H-Y)	No. 4 (I-E ^k)	H-2	H-Y
1	♀	(39)	T16H/X ^{Sxr}	31	9	1	2	5	16	16	ND	k	-
	♀	(30)	XX	21	2	1	1	5	15	2	ND	k	-
	♂	(46)	XY	22	-1	17	-1	121	ND	1	ND	k	+
2	♂	(59)	(T16H or X)/X ^{Sxr}	16	15	1	4	31	71	4	ND	k/b	-
	♂	(58)	T16H/Y	16	19	29	22	539	1,290	38	ND	k/b	+
	♀	(55)	XX	11	0	0	0	45	52	9	ND	k	-
3	♀	(64)	XX	ND	ND	ND	ND	99	70	10	ND	k	-
	♀	(68)	T16H/X ^{Sxr}	ND	ND	ND	ND	1,122	43	17	ND	k	+
	♀	(69)	T16H/X ^{Sxr}	ND	ND	ND	ND	813	16	16	ND	k	+
	♂	(71)	T16H/Y	ND	ND	ND	ND	1,245	1,358	346	ND	k/b	+
	♂	(72)	T16H/Y	ND	ND	ND	ND	1,271	60*	163	ND	k/b	+
	♂	(73)	XY	ND	ND	ND	ND	1,009	1,645	677	ND	k/b	+
	♂	(74)	XY	ND	ND	ND	ND	1,261	1,367	265	ND	k/b	+
4	♂	(117)	(T16H or X)/X ^{Sxr}	ND	ND	ND	ND	ND	20	2	ND	k/b	-
	♂	(114)	T16H/Y	ND	ND	ND	ND	ND	760	90	ND	k/b	+
	♀	(121)	XX	ND	ND	ND	ND	ND	6	1	ND	k/b	-
5	♂	(146)	(T16H or X)/X ^{Sxr}	40	TF	3	8	ND	ND	1	189	k	-
	♂	(CBA)	XY	49	TF	39	8	ND	ND	4	233	k	+
	♀	(CBA)	XX	30	TF	0	9	ND	ND	15	224	k	-
6	♀	(197)	T16H/X ^{Sxr}	41	3	11	7	5	ND	4	590	k	-
	♂	(CBA)	XY	40	0	36	0	449	ND	3	334	k	+
	♀	(CBA)	XX	40	1	7	3	6	ND	4	419	k	-

Spleen cells were obtained by partial splenectomy from number-coded mice. The code was broken only after the results had been computed. To prepare stimulator cells for the T-cell clones, or targets for cytotoxic T cells or antibody plus complement lysis, red blood cells were removed from the spleen cell suspensions, which were resuspended at 8×10^5 ml in RPMI 1640 containing 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES, penicillin 50 IU ml⁻¹ and streptomycin 50 µg ml⁻¹ (RPMI medium). Appropriate aliquots were taken for use as Con A blast targets (see below) and the cells to be used to stimulate the T-cell clones were irradiated (2,000 R) before being dispensed in volumes of 0.1 ml per well into flat-bottomed microtitre plates for the proliferation assay (see below). H-2 typing was performed with cytotoxic T cells (cell-mediated lysis, see below), and in experiments (5) and (6), with a proliferative T-cell clone specific for the I-E^k molecule. Typing was confirmed in experiments (2)-(6) using antibody plus rabbit complement with ⁵¹Cr-labelled splenic lymphocytes (either fresh, 2-day or 5-day Con A blasts) and monoclonal antibodies 28-13-3S (anti-K^b) and 12-2-2S (anti-K^d), each at a 1:7,500 dilution of ascites fluid (provided by Dr D. Sachs). A single-step cytotoxicity test was done in a final volume of 200 µl (50 µl cells, 50 µl antibody plus 100 µl rabbit complement at a final concentration of 1:30). Cell-mediated lysis (CML) was performed essentially as described in ref. 23 using as attacker cells 5-day mixed lymphocyte cultures (MLC) of the following type: anti-H-Y^b = C57BL/10 female immunized *in vivo* and boosted in MLC with C57BL/10 male spleen cells; anti-H-2^k = BALB.K or CBA anti-C57BL/10; anti-H-Y^k = (C57BL/10 $\times$ CBA)F₁ female immunized *in vivo* and boosted *in vitro* with CBA male cells; anti-H-2^k = C57BL/10 or BALB.B anti-CBA. Target cells were splenic lymphocytes held in culture before the CML assay either for 2 days with RPMI medium containing 4 µg ml⁻¹ Con A or for 5 days in RPMI medium with 1 µg ml⁻¹ Con A, and 25% rat spleen Con A supernatant as a source of interleukin-2 (IL-2). 2-day targets (in 2-ml volumes) were cultured in cluster plate wells at 5×10^5 ml⁻¹; for 5-day targets, 1×10^5 cells were cultured in 12.5-ml volumes in 50-ml tissue culture flasks kept upright. Target cells were labelled with ⁵¹Cr and 10⁴ cells per well were added in 100-µl volumes to round-bottomed microtitre wells in plates into which attacker cells had been titrated (100 µl volumes) in triplicate in four threefold dilutions, giving a starting (highest) attacker:target (A:T) cell ratio of ~30:1. Controls consisted of target cells incubated with 100 µl medium alone (medium control, C) and target cells with 100 µl 5% Triton X-100 (maximum release, M). After incubation for 3 h, the assay plates were centrifuged briefly, 100 µl of supernatant collected for γ counting. Per cent specific lysis was calculated as $E - C/M - C$ where $E =$ c.p.m. of target cells incubated with attacker cells, and C and M are the c.p.m. of target cells incubated with medium alone and with Triton X-100, respectively. Per cent lysis was calculated for each A:T ratio on each target, and regression analysis was performed for each attacker/target combination. The per cent lysis at A:T = 10:1 was computed from the regression curves; this value is given in the table. Positive values are defined as those obtained from titration data, with a correlation coefficient $r^2 \geq 0.80$; these values are italicized. Proliferative clones: C-3 and 10-2-12 have been used previously for H-Y typing²³, and are specific for D^k+H-Y and D^b+H-Y, respectively. These clones are IL-2-dependent, therefore IL-2 (rat spleen Con A supernatant) was included in the assays at 10-20%. Clones 10-2-12 and 2-1-1 have been described previously²⁸. Clone 2-1-1 is specific for I-A^b+H-Y and is IL-2 independent. Clone A-4, like C-3, recognizes D^k+H-Y and is IL-2-dependent (K.T., unpublished). Clone no. 4 recognizes the I-E^k molecule and is IL-2-independent (K.T., unpublished). Cells from these clones were used in the proliferation assays 12-14 days after their last *in vitro* restimulation. They were plated at 10^4 per cells per well together with either 8×10^5 stimulator cells per cell or medium alone. After 3 days of culture, clones 2-1-1 and no. 4 were pulsed with ³H-thymidine for 6 h; after 4 or 5 days of culture clones 10-2-12, C-3 and A-4 were similarly pulsed. DNA-bound thymidine was collected onto filters for β-counting. The results shown are an average of triplicate wells. Clone C-3 was used in experiments (1), (2) and (3), whereas clone A-4 was used in experiment (6), as it was growing better at the time. Insufficient C-3 cells were available for use in experiments (4) and (5). Background counts of cloned cells incubated in medium (plus IL-2 in the case of the IL-2-dependent clones C-3, A-4 and 10-2-12) without stimulator cells were <1,000 c.p.m. (normally <500 c.p.m.) in all experiments except (2) in which they were 2,372 for C-3. Counts of cloned cells incubated with female stimulator cells were sometimes higher than medium $\pm$ IL-2 alone and this may reflect 'feeder effects' $\pm$ back stimulation, since the clones in some cases expressed an H-2 type not present on the stimulator cells. Where the c.p.m. in the presence of female stimulator cells was high (as in expts (2) and (3) for clones C-3 and 10-2-12), that with male cells was very high indeed (more than an order of magnitude higher), so that discrimination between positive and negative was clear. The data shown for each experiment are restricted to mice shown in Fig. 1, plus additional controls where necessary to evaluate the data. However, each H-2 and H-Y typing experiment included generally at least 20 mice (for example, the data in expt (1) are taken from a much larger experiment published in full in ref. 23). To date, we have typed over 200 mice in this study; for all mice other than 39, 59, 117, 146 and 197 (all H-Y-negative although each carries *Sxr*) the H-Y typing with T-cell clones and CML was concordant with the genotype, that is, all males with a Y chromosome or X^{Sxr} were H-Y-positive, as were T16H/X^{Sxr} females. ND, not done. TF, technical failure: the anti-H-2^b attacker cells used in this experiment failed to lyse control C57BL/10 male and female targets.

* From the data using clone 10-2-12, mouse 72 is a presumed intra-H-2 recombinant and lacks the D^b molecule; serological typing shows that it expresses the K^b molecule, and the results with clone 2-1-1 show that it expresses the I-A^b molecule and is H-Y-positive. Clone C-3 confirms that mouse 72 is H-Y-positive and expresses D^k.

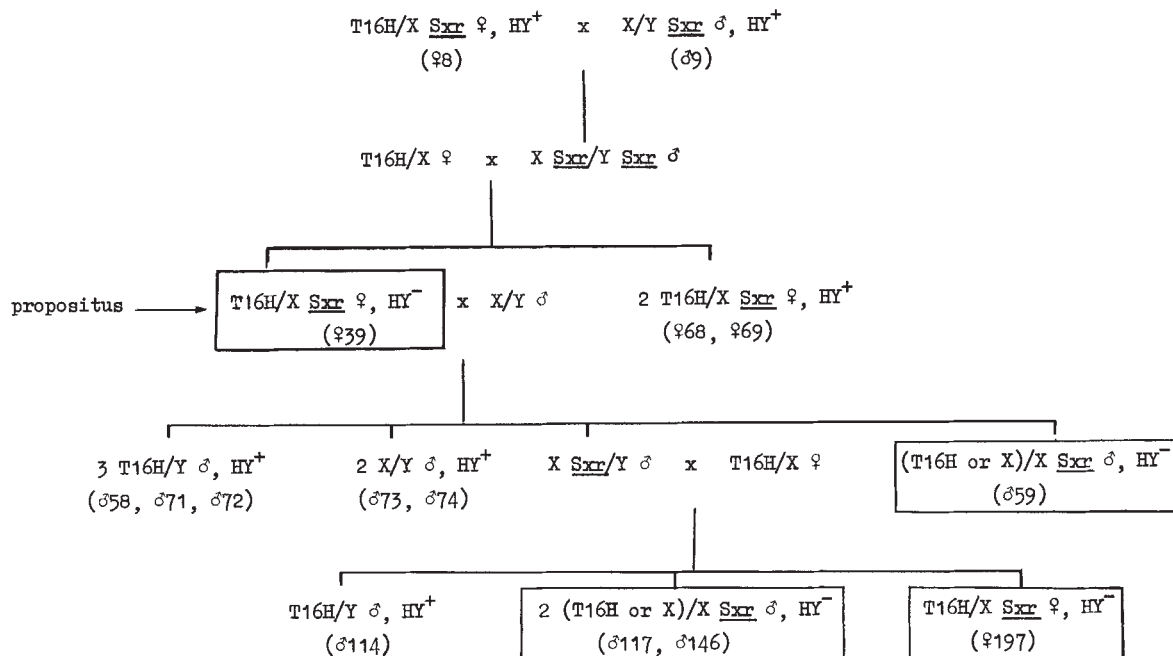


Fig. 2 Family tree of *Sxr*-carrying ♀39 (see Table 2 of Simpson *et al.*²³). The stocks of mice used and the production of T16H/X^{*Sxr*} females are described by McLaren and Monk¹⁹. T16H/X^{*Sxr*} females, X/Y^{*Sxr*} males and X^{*Sxr*}Y^{*Sxr*} males were identified by progeny testing. T16H/Y and X/X^{*Sxr*} males were distinguished from X/Y males and from each other by testis weights, combined where necessary with testis histology and often also with karyotyping. T16H/X^{*Sxr*} males were not distinguished from X/X^{*Sxr*} males. Only those mice tested for H-Y antigen have serial numbers. For details of H-Y antigen testing, see Table 1.

the alteration is stable, it should be detected in subsequent generations.

Table 1 shows the results of testing female 39 herself, one (T16H or X)/X^{*Sxr*} son, two (T16H or X)/X^{*Sxr*} grandsons and one T16H/X^{*Sxr*} granddaughter, together with appropriate control mice, using both cytotoxic T cells and H-Y-specific T-cell clones. In experiments (1), (2) and (6), H-2 type and H-Y type were assigned on the basis of the cytotoxicity assay, and independently confirmed by the clonal proliferation test. In experiments (3) and (4), H-2 and H-Y were assigned on the clonal test alone; in experiment (5), H-Y type was assigned by the cytotoxicity results, but the clonal test confirmed the H-2 typing. In addition, H-2 typing by monoclonal antibody plus complement was used to confirm the T-cell H-2 typing in each experiment except (1).

Details of the cytotoxic T-cell, clonal proliferation and antibody plus complement lysis assays are given in Table 1. Briefly, spleen cell suspensions were made from partial splenectomy samples of each mouse to be tested. After removal of red blood cells, the cell suspension was divided into two aliquots, one of which was placed in culture with concanavalin A (Con A) to generate blast cells for use as target cells 2 or 5 days later in both the cytotoxic T cell and antibody plus complement lysis assays. The other aliquot was irradiated and used to stimulate the T-cell clones. All the evidence is consistent with the H-Y antigen defined by cytotoxic T cells and H-Y-specific T-cell clones being the same as that defined by transplantation.

The family tree of ♀39 is shown in Fig. 2; it is apparent that all tested descendants of ♀39 who have inherited her mate's Y chromosome are, as expected, positive for H-Y antigen; on the other hand, those who have received her *Sxr* region in the absence of a Y chromosome, that is, X/X^{*Sxr*} males and T16H/X^{*Sxr*} individuals (whether male or female), are H-Y-negative. Histological examination of the testes of the three H-Y-negative (T16H or X)/X^{*Sxr*} males suggests that they do not differ from those of H-Y-positive (T16H or X)/X^{*Sxr*} males. It seems, therefore, that we are dealing with a variant form of the *Sxr* region, which retains the testis-determining property but has lost the sequences controlling the expression of H-Y trans-

plantation antigen. We propose that this variant be designated *Sxr'*, with the constitution *Tdy*⁺, *Hya*⁻, where *Hya* is the locus (structural or regulatory) controlling the expression of H-Y antigen. *Tdy* and *Hya* may be the same locus, or two separate loci.

Our data do not allow us to determine whether *Sxr'* lacks the *Hya* locus altogether, or retains it in a modified form such that the antigenicity by which the molecule is defined has been lost. In either case, the separation of testis determination from expression of H-Y transplantation antigen eliminates this antigenic determinant as a candidate for the primary sex determinant. Previous reports point to the same conclusion. A single sterile X0 male described by Melvold *et al.*²⁴ had testes but was negative for H-Y antigen; while many H-Y-positive females have been described, not only the presumed mosaic T16H/X^{*Sxr*} females mentioned above²³, but also several XY females reported by Eicher and colleagues^{25,26}.

If mice lacking the H-Y transplantation antigen can develop as phenotypic males, what is the function of the H-Y antigen in normal males? Such a ubiquitous substance, present as far as we know on all cells of all male mice examined, except for those described here and the single male described by Melvold and colleagues²⁴, should be of fundamental significance. We are examining the possibility that H-Y is required for some aspect of male reproduction, for example spermatogenesis. Any such defect would not manifest itself in our H-Y-negative males, as they are sterile, with germ cells that degenerate soon after birth and do not undergo spermatogenesis, because of the presence of two X chromosomes.

We do not know whether the event giving rise to the variant *Sxr'* region occurred in the cell lineage of the spermatozoon contributing to ♀39, or in a previous generation. However, it may be no coincidence that ♀39 was the daughter of a homozygous X^{*Sxr*}/Y^{*Sxr*} male (Fig. 2), because the presence of two homologous *Sxr* regions, containing copious repeated DNA sequences²⁷, would be expected to facilitate unequal crossing-over and hence separation of *Hya* and *Tdy*, if they are indeed two separate loci, or loss of part of *Hya*. We have so far examined 65 progeny of homozygous and 99 progeny of heterozygous *Sxr*

males, without detecting another such event. The unequal crossing-over model makes no prediction as to the order of *Hya* and *Tdy* within the *Sxr* region, but requires that the *Hya* locus is flanked on both sides by homologous sequences.

Note added in proof: We have continued the pedigree shown in Fig. 2 to five generations ($\varnothing 39$ =generation 0). Of 19 (T16H or X)/X^{Sxr} males and 2 T16H/X^{Sxr} females typed so far in this pedigree, all have proved HY negative.

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Analysis of persistent virus infections by *in situ* hybridization to whole-mouse sections

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Nucleic acid hybridization techniques have contributed significantly to the understanding of gene organization, regulation and expression¹⁻⁴. In the context of persistent or latent viral infections, hybridization with specific labelled probes represents the most sensitive assay presently available for detection of viral genomes⁵⁻⁸. During the course of persistence, viral genomes may exist in multiple and yet quite segregated areas in an infected host, but examination of all tissues remains difficult and time-consuming. At present, the application of hybridization assays to *in vivo* infections requires either chemical extraction of nucleic acid coupled with dot-blot⁹ and gel transfer¹ techniques or *in situ* hybridization^{5,10} to cryostat tissue sections. In both cases, selected tissues must be removed by dissection before analysis. We describe here a procedure which allows efficient and reproducible screening of all tissue in an infected host. Our technique allows detection of viral genetic material in whole-body sections of infected mice, and provides the first evidence *in vivo* for accumulation of viral genetic material with a parallel decrease in infectious virus during persistent virus infection. This technique should be widely applicable to studies of developmental regulation of gene expression, for monitoring locations of gene expression in transgenic mice and for analysis of molecular mechanisms in pathogenesis.

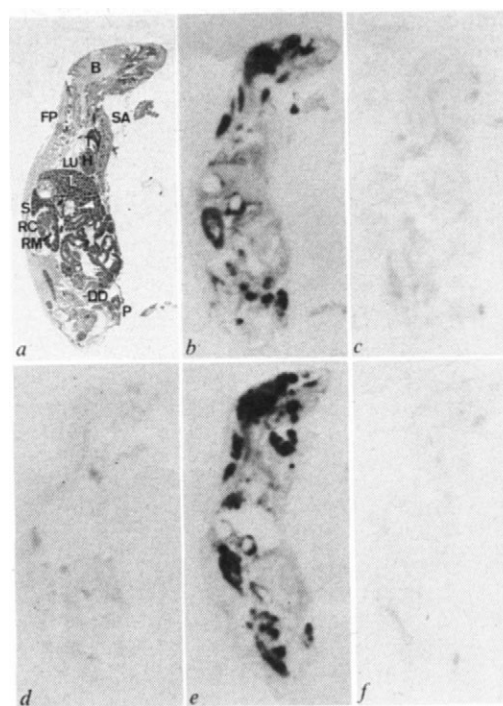


Fig. 1 *In situ* hybridization to whole-body sections of a persistently infected SWR/J mouse and age- and sex-matched controls. *a, b, f*, Sections from a persistently infected 12-week-old mouse; *a* and *b* are different representations of the same 40-μm section. Histopathological details are recorded in *a* and relate to: B, brain; SA, salivary gland; FP, brown fat pad; T, thymus; H, heart; LU, lung; L, liver; S, spleen; RC, renal cortex; RM, renal medulla; DD, ductus deferens; P, penis. The arrow indicates the mucosa of the stomach. *b* Shows the corresponding pattern of tissue hybridization with a cloned cDNA probe derived from the S RNA segment of LCMV: Armstrong CA 1371. *c*, Hybridization of the LCMV S cDNA probe to an uninfected control mouse. *d, e*, Adjacent 40-μm sections from a 10-week-old infected mouse; *d*, hybridization with a cytomegalovirus probe from the immediate-early region²⁵; *e*, hybridization with the cloned LCMV S cDNA probe. *f*, The effect of treating a section with RNase before hybridization with the LCMV probe. *In situ* hybridization to SWR/J mice is also shown in Fig. 3 where a moderate hybridization signal is seen for the livers; this level of signal is typical of SWR/J liver tissue and the liver signal in Fig. 1 is unusually low.

Methods: Mice were killed with ether, exsanguinated, shaved and then frozen in blocks of 2.5% carboxymethyl cellulose (CMC) by slow immersion in dry ice ethanol. These blocks could be stored at -30 °C for several weeks with no apparent effect on signal intensities. The CMC blocks were rapidly cut to the relevant planes on an LKB cryomicrotome, then sequential 40-μm sections were collected on Scotch tape. With the tape firmly pressed against the CMC block, the attached section could be lifted away as the block was moved under the microtome blade. The sections were air-dried, fixed by immersion in freshly prepared 4% paraformaldehyde (in phosphate-buffered saline, PBS), rinsed twice in PBS and again air-dried. Pairs of sections were usually hybridized in Seal-A-Meal bags. Wetting the tape allows easy transfer of the sections to the bags. Prehybridization was performed at 37 °C for 12-24 h in 50% deionized formamide, 5× SSC, 2.5× Denhardt's solution with 100 μg ml⁻¹ boiled, sonicated salmon sperm carrier DNA; 6 ml of solution were used for each pair of sections. Hybridization was performed at 37 °C for 60-68 h using 2 ml of the same solution for each pair of sections and 2-4 × 10⁶ c.p.m. of ³²P-labelled nick-translated DNA probe, specific activity 1-5 × 10⁸ c.p.m. per μg DNA. In all cases, the LCMV cDNA inserts were excised from plasmid vectors and purified by gel electrophoresis before labelling. The average fragment length of the denatured probe was 200-400 bases. The sections were washed three times for 30 min each at 37 °C in 2× SSC, and once for 30 min at 55 °C in 2× SSC. After a final 30 min wash at 55 °C in 0.1× SSC, the sections were stained with haematoxylin-eosin (modified to substitute lithium carbonate for the ammonium carbonate wash) and exposed at -70 °C using Kodak XAR-5 film and a Cronex Lightning Fast intensification screen. The film was developed after 20 h exposure.

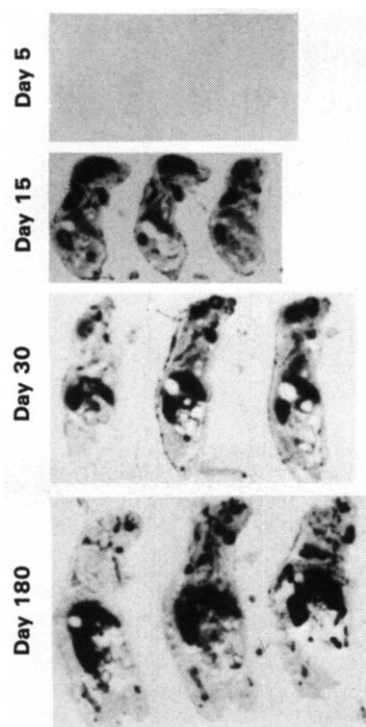


Fig. 2 BALB/WEHI mice infected at birth with LCMV (60 PFU given intracerebrally) were killed at the indicated times. Whole-body sections from individual mice were hybridized with a cloned cDNA probe derived from part of the LCMV S genomic RNA segment (see Fig. 1 legend and ref. 17 for details). This study shows *in situ* hybridization with sections from different animals at different time points (four mice at day 5, three mice at days 15, 30 and 180). Data representative of 17 mice studied. Hybridization to the skin (visible especially with the day 30 animals) is not normally seen.

Inoculation of newborn mice with lymphocytic choriomeningitis virus (LCMV) readily leads to persistent virus infection¹¹⁻¹⁴. Following infection, mice of most inbred strains reach maturity but retain life-long viraemia, with infectious virus present in blood and major organs. The genome of LCMV comprises two single-stranded RNA segments of negative polarity (RNAs designated L and S, approximate lengths 9 and 4 kilobases (kb), respectively) that exist as ribonuclear protein complexes within infected cells and virions^{15,16}. We have prepared and characterized partial complementary DNA clones to both the L and S genomic RNA segments¹⁷. These clones have been labelled with ³²P by nick-translation¹⁸ for use as hybridization probes¹⁹.

Typical results from *in situ* hybridization are shown in Fig. 1, where the contrast between positive and negative controls is clear. The specificity of the hybridization reaction was verified by three independent approaches in several experiments. There was no positive signal from sections of uninfected, age- and sex-matched control animals that were hybridized with the LCMV probes (Fig. 1c) and pretreatment of LCMV-infected whole-mouse sections with RNase effectively abolished the hybridization signal (Fig. 1f). Sections from LCMV-infected animals that were hybridized with a probe from an unrelated virus (human cytomegalovirus) also failed to show any signal (Fig. 1d). There was good tissue preservation during the experimental manipulations and, after hybridization, sections could be treated with histological stains (Fig. 1a, b) before autoradiographic exposure to facilitate tissue identification. Obviously, any single section may not include all tissue types, but serial sections were made at different depths throughout the animal

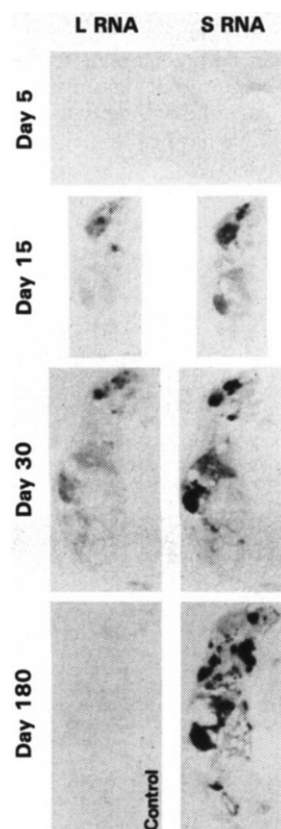


Fig. 3 SWR/J mice infected at birth with LCMV (60 PFU given intracerebrally) were killed at various ages. Whole-body sections from individual mice were hybridized with cloned LCMV cDNA probes to part of either the large (L) RNA or small (S) genomic RNA segments. Two mice are shown for day 5. The control is an uninfected mouse section reacted with the ³²P-labelled L RNA probe. This study shows similar distribution of L and S genetic material in different tissues during infection (the distribution of L RNA in tissues from the 180-day-old mouse was similar to that shown with the S probe). The data are representative of >20 individual SWR/J mice studied.

to ensure sampling of all the major tissues at various planes. This whole-body sectioning revealed viral genetic material in unexpected locations, for example, the mucosa of the stomach (Fig. 1, arrowhead) and the ductus deferens (Fig. 1, DD). Other experiments using a cDNA probe derived from 28S ribosomal RNA (P.J.S., unpublished results) showed positive hybridization to many tissues, with the strongest signal exhibited by thymus (data not shown).

During these studies we compared several support substances, tissue fixatives and detergent treatments in an attempt to optimize the hybridization procedure. We found that picking up tissue on 3M Scotch tape was preferable to using Gene Screen Plus (NEN), nitrocellulose (Schleicher & Schuell) or a glass slide. Gene Screen Plus was similar in signal-to-noise ratio and tissue retention to 3M tape, but staining with haematoxylin and eosin for morphological details was unsatisfactory. Hybridization to sections on nitrocellulose showed a 5-10-fold signal reduction and, after baking at 80°C, there was an increased tendency to tissue loss during the hybridization procedure. Sections picked up on glass slides varied in terms of tissue retention. Multiple procedures using proteases and Nonidet-P40 showed no significant enhancement of the hybridization signal. We usually cut 40-µm sections for hybridization, but some were as thin as 15-µm. Once cut and fixed, the sections could be stored dry for up to 3 weeks with no loss of hybridization signal. Of various fixatives we have tried, 4% paraformaldehyde seemed the most effective.

Figure 2 illustrates the distribution of viral genetic material during the lifespan of persistently infected BALB/WEHI mice. The patterns of hybridization are highly reproducible for

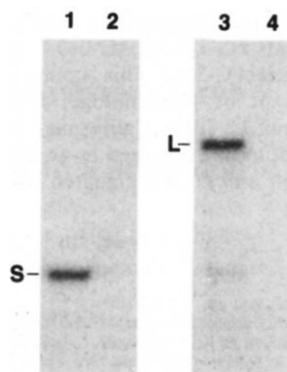


Fig. 4 Total cellular RNA from LCMV-infected or uninfected L-929 cells was extracted by the guanidinium thiocyanate procedure²⁶, denatured with glyoxal, separated by agarose gel electrophoresis, then transferred to a nitrocellulose filter²⁷. The filter was hybridized with ³²P-labelled probes as described for the mouse section hybridization experiments. Lane 1, RNA (3 µg) from L-929 cells persistently infected with LCMV. Lane 2, RNA (3 µg) from a parallel culture of uninfected L-929 cells; the probe was an S-specific cDNA clone. Lanes 3, 4, the same nitrocellulose filter after stringent washing and rehybridization with an L-specific cDNA clone. The apparent hybridization of the L probe to an 'S-sized' RNA species is being investigated.

individual mice at each different time. Viral RNA was readily detectable in sections made 15 days after birth but sections made 5 days after birth showed a barely detectable hybridization signal, although infectious virus can be isolated from tissues and blood of infected animals at this time^{13,14}. With increasing age, there was no consistent change in the distribution of viral RNA when comparing persistently infected BALB mice at 15, 30 or 180 days. The titre of infectious LCMV recovered from the brains of individual BALB/WEHI mice 5 days after initiating infection in plaque-forming units (PFU) per g (mean for five mice: 6×10^6 , range 7×10^5 – 9×10^6) was over 2,000-fold higher than that obtained from mice at 180 days (mean for five mice: 3.0×10^3 , range 1×10^3 – 7×10^3), yet the accumulation of viral nucleic acid sequences was marked at 180 days and insignificant at 5 days. The virus titre at 15 and 30 days after infection was 1.4×10^5 and 2.5×10^4 , respectively (values represent the mean for five animals). In all cases, virus titres were measured in plaque assays using Vero cell monolayers in standard conditions¹⁴. These experiments showing increased levels of viral nucleic acid accompanied by a significant reduction in the titre of infectious virus in brain tissue, were duplicated over the course of persistent infection in a different strain of mouse, SWR/J (see Fig. 3). The titre of infectious LCMV (PFU per g) recovered from the brains of individual SWR/J mice 5 days after initiating infection was 2.2×10^7 (mean for four animals, range 2×10^6 – 4×10^7) and at 180 days the titre was 2.5×10^4 (mean for five animals, range 7×10^3 – 8×10^4). The apparent discrepancy between increased levels of viral nucleic acid and reduced titres of infectious virus suggests the presence of defective particles generated during the course of natural infection, a possibility that we are investigating. This theoretical possibility has been raised by others^{20,21} primarily on the basis of experiments with cultured cells.

Comparison of sections from persistently infected BALB/WEHI mice (Fig. 2) and SWR/J mice (Figs 1, 3) indicated that there was a significant difference in the levels of LCMV nucleic acid sequences in the liver whereas other tissues were similar in both mouse strains. For BALB/WEHI mice, 15 of 17 animals showed strong hybridization to liver whereas equivalent strong hybridization was seen in only 3 of 20 SWR/J mice.

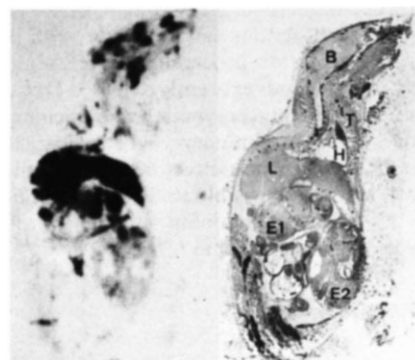


Fig. 5 Whole-body *in situ* hybridization and histopathological analysis of a single section taken from a persistently infected, pregnant BALB/WEHI mouse 19–20 days after conception. This female mouse had been mated with a persistently infected BALB/WEHI male. *In situ* hybridization was performed as described for Fig. 1, using a cloned cDNA probe derived from part of the LCMV L RNA. Two embryos can be observed in the stained section. There is a strong hybridization signal from the liver (L) and brain (B) of the pregnant mouse. The area of strong hybridization above the thymus in the pregnant mouse is from the salivary gland. H, heart; E1, embryo 1; E2 embryo 2. The hybridization pattern to embryo 1 is highly suggestive of a central nervous system location for viral genetic material and this is verified by the accompanying histopathology. Similar results were obtained with two additional congenitally infected pregnant mice using an LCMV S-specific probe.

In the next series of experiments, we analysed adjacent 40-µm sections from SWR/J mice during the course of persistent infection, using equivalent probes derived from the L and S genomic segments of LCMV (Fig. 3). There was no difference in the tissue patterns of hybridization for the L and S probes. The signal from L probes was normally about half that from S probes, consistent with our analysis of total RNA extracted from LCMV-infected tissue culture cells (data not shown). Figure 4 shows the specificity of the L and S RNA probes.

The sensitivity of the *in situ* hybridization technique has been assessed by dot-blot experiments⁹ using cloned LCMV cDNAs as standards. We have excised selected tissue areas from 40-µm sections and extracted total nucleic acid by SDS/proteinase K and phenol treatments to provide a direct measure of the LCMV RNA content in the section. For example, by hybridization with an S-specific cDNA probe, we estimate that there are ~250 pg of 'S-derived' RNA in the kidney sections of 30-day-old BALB/WEHI and SWR/J mice (Figs 2, 3). On this basis and together with data from other tissues, we calculate that the sensitivity of hybridization to tissue sections is reduced 2–10-fold relative to the dot-blot method. We are currently investigating several parameters to increase the sensitivity of the technique by enhancing the specific activity of the probes using strand-specific probes from M13 (ref. 22) or SP6 (ref. 23) vectors. Results reported here were obtained with ³²P probes of specific activity 1 – 5×10^8 c.p.m. µg⁻¹.

Whole-body *in situ* hybridization proved useful in studying the tissues of persistently infected pregnant mice. Figure 5 shows viral genetic material in congenitally infected embryos of a pregnant, persistently infected BALB/WEHI mouse. One embryo (Fig. 5, E1) shows a heavier load of viral RNA than the other (Fig. 5, E2). Studies of other congenitally infected pregnant mice revealed similar uneven accumulation of viral nucleic acid sequences among the carrier embryos. We are currently evaluating the expression of LCMV genetic material in developing embryos, from gestation until after delivery, and the expression of developmentally regulated genes like those associated with myelination.

Thus, whole-animal sections can now be studied by *in situ* hybridization and locations and changes in viral gene expression can be mapped. Several limitations of the conventional *in situ*

hybridization procedure are largely avoided. There is good preservation of tissue and sections can be treated with histological stains after the hybridization procedure. Concurrent with these studies, Dubensky *et al.*²⁴ have recently detected DNA and RNA in organs of mice following polyoma and vesicular stomatitis virus infections. Finally, preliminary results indicate that LCMV antigens can be detected in mouse sections using immunological reagents. Hence, it is now possible to detect expression of viral RNA and protein while maintaining suitable morphology for detailed study at the whole-animal level.

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Serine- and threonine-specific protein kinase activities of purified gag-mil and gag-raf proteins

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Retroviruses carry cell-derived oncogenes (*v-onc*) that have the potential to transform cells in culture and induce tumours *in vivo*^{1,2}. One of the few carcinoma-inducing viruses is the acutely transforming retrovirus MH2 (refs 2, 3), which carries the putative oncogene *v-mil* and the known oncogene *v-myc* (refs 4-6). Recently, a high degree of homology was discovered between *v-mil* and *v-raf* (ref. 7), the transforming gene of the murine retrovirus 3611 murine sarcoma virus (MSV)⁸, whereas homology to *v-src* is low⁹. Both viruses express their oncogenes as the gag-fusion polypeptides^{6,10} p100^{gag-mil} and p75^{gag-raf} (of respective relative molecular mass (*M_r*) 100,000 and 75,000), while the *myc* oncogene of MH2 is expressed by means of a subgenomic messenger RNA¹¹. We have recently demonstrated that p100^{gag-mil} is not a nuclear protein⁶. Here we report that purified p100^{gag-mil} and p75^{gag-raf} exhibit protein kinase activities *in vitro* which, in contrast to the *src*-related p130^{gag-fps} of Fujinami sarcoma virus (FSV)¹² and all other characterized oncogene-encoded protein kinases, phosphorylate serine and threonine but not tyrosine. Both types of protein kinases phosphorylate lipids *in vitro*.

Previously reported protein kinase studies of p75^{gag-raf} have involved immunoprecipitation analyses. We have recently been able to recover highly purified and enzymatically active p130^{gag-fps} protein kinase from FSV-transformed cells¹³⁻¹⁶ (P. Donner *et al.*, manuscript in preparation), and have applied our protocol here to investigate whether p100^{gag-mil} and p75^{gag-raf} possess protein kinase activity. Immunoaffinity purification of p100^{gag-mil} and p130^{gag-fps} was performed by using monoclonal immunoglobulins (IgG) against p19, the amino-terminus of the gag portion¹⁷. ³⁵S-methionine-labelled cellular extracts of FSV- and MH2-transformed cells were applied to the columns to purify p100^{gag-mil} and p130^{gag-fps} (Fig. 1). As a control, p110^{gag-myc} from MC29-transformed quail fibroblasts was also purified

(p110, Fig. 1). Purification efficiency by this procedure is about 3,000-fold¹⁷.

p75^{gag-raf} carries a murine viral gag protein and cannot be isolated using the column described above. Therefore, IgG specific for the murine viral structural protein p30 was purified from polyvalent anti-p30 rabbit serum and used to set up an immunoaffinity column. p75^{gag-raf} was isolated from ³⁵S-methionine-labelled 3611 MSV-transformed rat fibroblasts. 3611 MSV codes for a doublet consisting of a glycoprotein of *M_r* 90,000 (gp90^{gag-raf}) and p75^{gag-raf} (ref. 8). Figure 1 shows the purified proteins. Two additional bands were detected which have been observed previously in immunoprecipitates and are considered to be gag-processing proteins¹⁰.

Both types of immunoaffinity columns were treated with 0.5% SDS after elution of the ³⁵S-methionine-labelled gag-fusion proteins so as to isolate possible residual proteins remaining on the immunobeads. No such proteins were detected (data not shown). Similarly, when aliquots of the immunoaffinity columns loaded with the ³⁵S-methionine-labelled gag-fusion proteins were analysed by polyacrylamide gel electrophoresis in analogy to the protein kinase experiments described below, no protein contamination was detected (data not shown).

The proteins recovered from ³⁵S-methionine-labelled cellular extracts showed no protein kinase activity. Enzymatically active proteins were recovered only from unlabelled cellular lysates applied to the immunoaffinity columns using a modified approach. Instead of being eluted from the columns, the proteins were allowed to remain attached to the immunobeads for protein kinase assay. The assays used 10 mM Mg²⁺ and 10 mM Mn²⁺ as divalent cations (Fig. 2); the optimum ion concentration has been determined to be 10 mM Mn²⁺ in all cases (data not shown). Autophosphorylation of p100^{gag-mil}, p75^{gag-raf}, and p130^{gag-fps} was detected, but no autophosphorylation occurred with gp90^{gag-raf} or p110^{gag-myc}. In the case of 3611, two additional bands of lower relative molecular mass were phosphorylated. Preliminary evidence indicates that these bands are the result of proteolytic processing¹⁰. The absence of protein kinase from MC29-transformed quail fibroblasts after even longer exposure times indicates that the p100^{gag-mil} protein kinase activity is not of normal quail cell origin.

An additional control experiment was performed to exclude the possibility that the serine/threonine protein kinase was of rat cell origin. 3611 MSV cells were passed over an anti-p19 column as a nonspecific IgG-containing column. No protein kinase activity was detected (Fig. 2a, control). The autophosphorylation reactions were standardized to identical protein

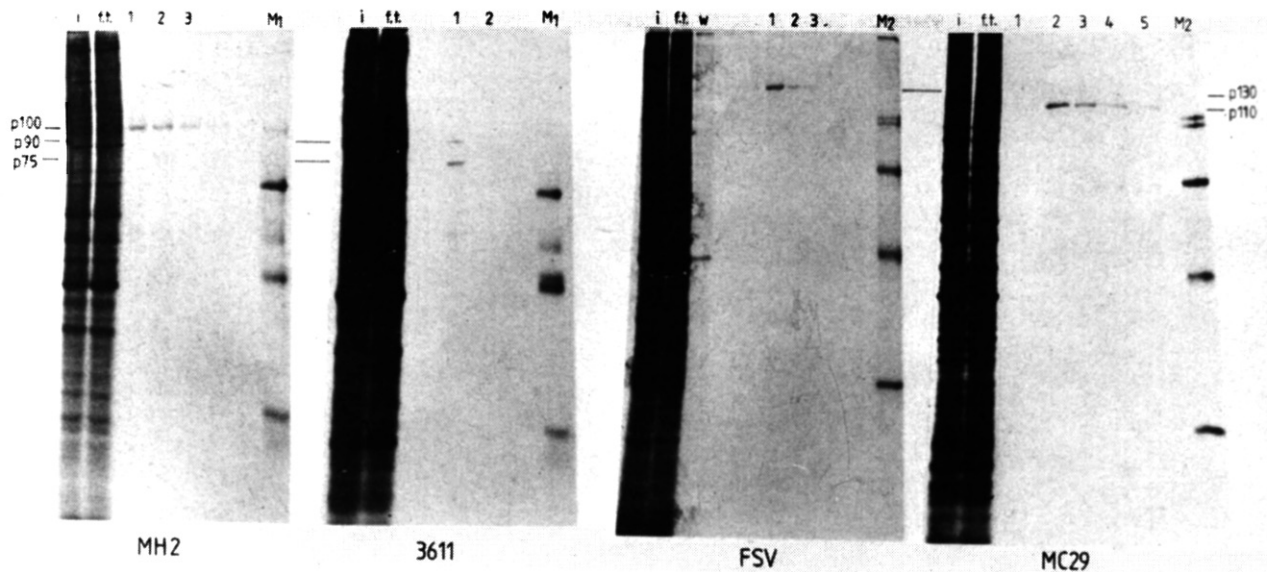
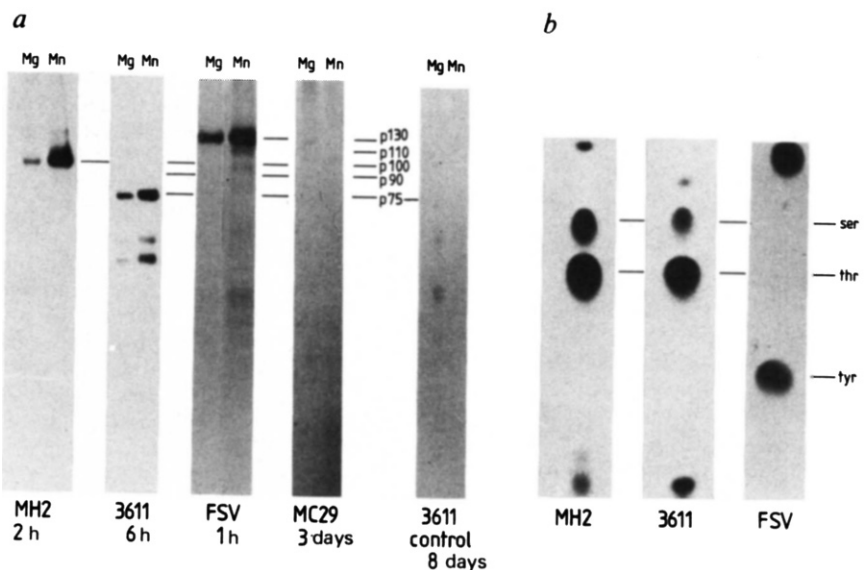


Fig. 1 Immunoaffinity purification of p100^{gag-mil}, p75^{gag-raf}, p130^{gag-fps}, and p110^{gag-myc} proteins. i, Input; ft, flow through; lanes 1-5, eluted fractions; M₁, M₂, size markers ($M_r \times 10^{-3}$) from top to bottom: M₁, 92, 69, 55, 46, 30; M₂, 92, 69, 46, 30. Specific activities of the eluted proteins were: p90/75^{gag-raf}, 8×10^4 c.p.m. μg^{-1} ; p130^{gag-fps}, 5×10^4 c.p.m. μg^{-1} ; p100^{gag-mil}, 3.6×10^4 c.p.m. μg^{-1} ; p110^{gag-myc}, 6×10^4 c.p.m. μg^{-1} . **Methods:** The cell lines used in this study were MH2-transformed non-producer quail fibroblasts⁸, 3611 MSV-transformed non-producer rat fibroblasts⁸, FSV-transformed rat fibroblasts¹² and MC29-transformed non-producer quail fibroblasts¹⁷. 5×10^7 cells of each of the cell types were labelled with ³⁵S-methionine (250 $\mu\text{Ci ml}^{-1}$) at 80% confluency for 2 h, lysed with 10 ml RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton X-100), 10 U ml⁻¹ Trasylol, centrifuged (10,000 r.p.m., 30 min, 4°C) and applied to immunoaffinity columns containing IgG against the murine viral protein p30 in the case of 3611, and against p19 for the other cell types. The anti-p30 column was prepared as follows: p30 was isolated by phosphocellulose column chromatography from 50 mg of MSV using elution conditions described elsewhere²³. Isolated p30 (1 mg) was coupled to activated CH-Sepharose 4B (Pharmacia). Goat anti-p30 serum (10 ml) was passed over the p30 column and specific IgG recovered. This anti-p30 IgG (2 mg) was then coupled to protein A-Sepharose 4B (0.2 g) as described elsewhere¹⁷ and used as anti-p30 immunoaffinity column. The anti-p19 immunoaffinity column has been described elsewhere¹⁷. After extensive washing, the purified proteins were eluted with citric acid buffer, pH 3.5, which was neutralized immediately. The eluted proteins (30 μl per 2 ml of each fraction) were analysed by gel electrophoresis and autoradiography.

Fig. 2 a, Protein kinase assay of purified proteins. b, Phosphoamino acid analysis.

Methods: a, 2×10^7 cells of MH2-, 3611-, FSV- and MC29-transformed non-producer fibroblast lines each were lysed with RIPA buffer in the presence of 1 mM dithiothreitol, centrifuged (10,000 r.p.m., 30 min, 4°C) and applied to immunoaffinity columns (200 μl of packed beads each) as described in Fig. 1 legend. 3611 lysates were also applied to an anti-p19 immunoaffinity column (3611 control). The columns were washed extensively¹⁷. Instead of eluting the proteins from the columns, they remained immobilized on the beads and were distributed to reaction tubes for further analysis. Protein kinase activities were tested in a total volume of 50 μl containing 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, supplemented with 10 mM MgCl₂ (Mg) or 10 mM MnCl₂ (Mn). 5 μl enzyme-coupled beads were used per reaction. Incubation time was 10 min at 37°C. 20 μCi of [γ -³²P]ATP (3,000 Ci mmol⁻¹) were used per assay. Reactions were terminated by the addition of four-fold concentrated sample buffer¹⁷ for SDS-polyacrylamide gel electrophoretic analysis. Samples were treated for 1 min at 100°C and applied directly to the gels. The gels were dried and exposed for autoradiography for the indicated periods of time. b, The autophosphorylated proteins were cut out of the gels, eluted and processed for phosphoamino acid analysis as reported previously¹⁸. Exposure time was 3-5 days at -70°C using intensifier screens.



contents of the lysate inputs. The immunoaffinity columns contained excess IgG and bound all available gag-fusion proteins. Based on these findings, we determined the radioactivity incorporated into the individual bands and found that p130^{gag-fps} incorporated fivefold more radioactivity than did p100^{gag-mil} or p75^{gag-raf}.

The phosphoamino acid content of the autophosphorylated proteins was determined¹⁸. Figure 2b shows that p100^{gag-mil} and p75^{gag-raf} are not tyrosine-specific protein kinases like p130^{gag-fps}, but phosphorylate serine and threonine instead. As both

p130^{gag-fps} and p75^{gag-raf} originate from rat cells¹², the differences in amino acid phosphorylation rule out protein kinase contamination by rat cells.

p75^{gag-raf} lacks the tyrosine acceptor site found in src¹⁹. It was therefore important to analyse the amino acid specificities of p100^{gag-mil} and p75^{gag-raf} not only in autophosphorylation reactions but also with exogenous substrates. For this we used rabbit muscle actin, calf thymus arginine- and lysine-rich histones, chicken H5, and phosvitin; casein was not used because of its high degree of autophosphorylation. The three protein kinases

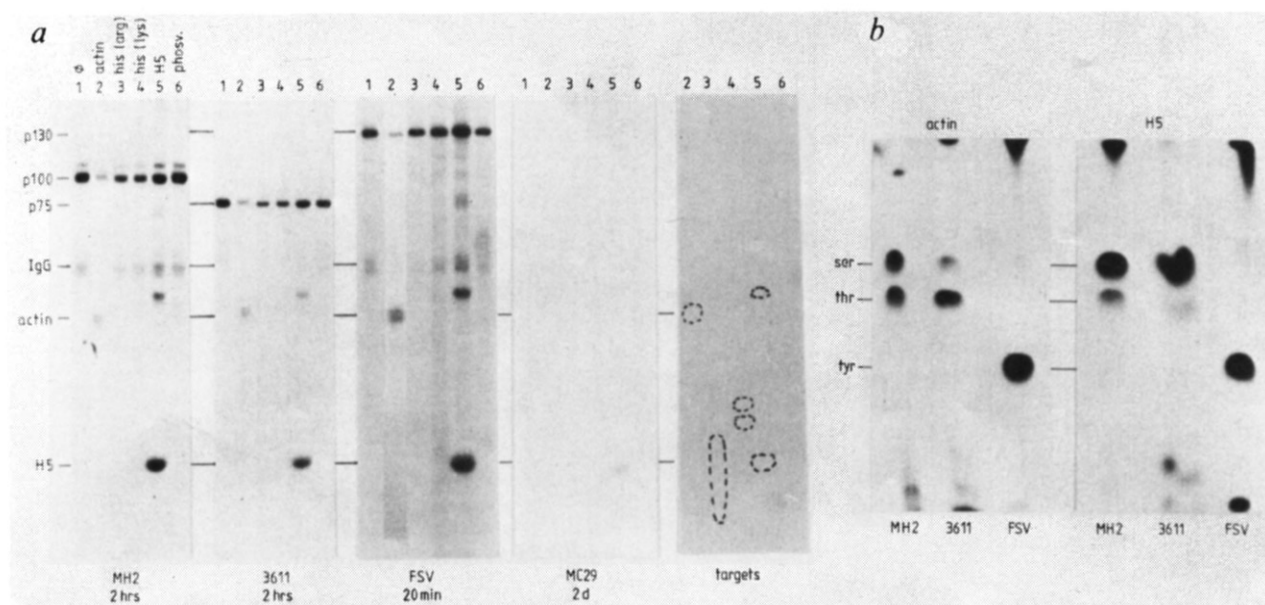


Fig. 3 Phosphorylation of substrates *in vitro*. *a*, Immunobead-immobilized enzymes were tested for their ability to phosphorylate exogenous substrates (5 μ g each, all from Sigma) *in vitro*. Protein kinase assays were performed as described in Fig. 2 legend in the presence of 10 mM $MnCl_2$. Lanes 1, no substrate; 2, actin; 3, histones, arginine-rich [his(arg)]; 4, histones, lysine-rich [his(lys)]; 5, chicken histone H5; 6, phosvitin. For control, all substrates were tested for autophosphorylation in the absence of kinases. Exposure time was as indicated using intensifier screens at $-70^\circ C$. The dotted circles indicate where the substrates migrated as determined by Coomassie blue staining. *b*, Phosphoamino acid analysis of the two substrates actin and H5 phosphorylated by MH2, 3611 and FSV protein kinases. The phosphoproteins were cut out of the gel shown in *a* and processed as described previously¹⁸. For exposure time, see Fig. 2b legend.

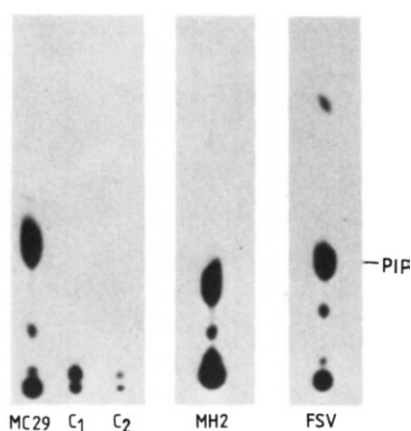


Fig. 4 TLC analyses of lipids radiolabelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Immunobead-immobilized protein kinases from MH2-, FSV- and MC29-transformed cells were incubated with detergent-treated L- α -phosphatidylinositol 4-monophosphate. Details of the conditions have been published elsewhere¹⁴. Abbreviation: PIP, L- α -phosphatidylinositol; C1, control in which lipid was omitted; C2, control in which protein was omitted. PIP marker was co-chromatographed and traced by fluorescent light. Exposure time was 3 days at $-70^\circ C$ using intensifier screens.

phosphorylated histone H5 and actin, but phosphorylation of the other substrates was undetectable (Fig. 3a). When phosphoamino acid analysis was performed with the phosphorylated actin and H5 target molecules (Fig. 3b), p100^{gag-mil} and p75^{gag-raf} produced serine and threonine phosphorylation, while p130^{gag-fps} phosphorylated tyrosine. Some IgG also became phosphorylated. Actin seemed to inhibit autophosphorylation, while cyclic AMP inhibited all three protein kinase activities at concentrations ranging from 5 μ M to 0.5 mM (data not shown).

The recent finding that two src-related tyrosine protein kinases show lipid-phosphorylating activity^{20,21} raised the question of whether the protein kinases described here, although lacking tyrosine specificity, are also lipid kinases. Figure 4 shows that p100^{gag-mil} and p130^{gag-fps} indeed phosphorylate lipids *in vitro*. However, MC29 and normal quail cells processed by anti-p19 immunofluorescence chromatography resulted in similar levels of lipid phosphorylation under identical assay conditions (see MC29, Fig. 4).

Our results describe for the first time the presence of a serine/threonine protein kinase activity in two purified oncogene protein products. This activity is absent from quail and rat cell lines transformed by two other oncogenes, indicating that the kinase is not of cellular origin. Our results demonstrate that tyrosine phosphorylation can no longer be considered as a definitive property of oncogene-specific protein kinases. The recently observed lipid phosphorylation through the action of src²⁰ and ros²¹ oncogenes does not appear to be an intrinsic property of the protein kinases described here since similar levels of activity were also found associated with p110^{gag-myc} and normal cell controls. Phosphorylation of lipids or proteins as potential target molecules needs further evaluation. Indirect evidence suggests that the serine/threonine protein kinases described here may not be unique to mil/raf but may also be characteristic of the mos oncogene²².

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Afocal apposition optics in butterfly eyes

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In most apposition compound eyes there are two components to the optical system of the ommatidium^{1,2}, the cornea and the crystalline cone. The focusing power of the cornea is well documented^{3,4} whereas the crystalline cone is usually regarded as a mere optical spacer^{5,6}; consequently, the ommatidial optics will consist of a simple focusing lens. To the contrary, we now demonstrate the existence of a complete afocal telescope in each ommatidium of butterfly apposition eyes. The optical system is an extreme variant of that found in refracting superposition eyes, thereby providing a connection between butterflies and moths.

The apposition eye consists of optically isolated units (ommatidia) each having a lens forming an inverted image (see Fig. 1 of ref. 7). In superposition eyes, the lens systems of many ommatidia cooperate to form a superimposed erect image.

Here we measured the optical properties of the ommatidia in the eye of a common Australian nymphalid butterfly, *Heteronympha merope*. The focal length, f , of the cornea (posterior nodal distance) was determined by suspending a cleaned corneal cup from a hanging drop of Ringer's solution, and measuring the size of the image of a distant object viewed through the cornea; this gave a focal length of $48.6 \pm 1.2 \mu\text{m}$ ($\pm$ s.d., $N = 10$). The distance from the cornea to the image ($f' = nf$) is $67.3\text{--}70.7 \mu\text{m}$. (The refractive index used here, $n = 1.42$, is an average derived from cornea (1.52), corneal process (1.34) and cone (1.41).) In sectioned material the rhabdom tips are consistently found at a depth of $70 \mu\text{m}$, so that if the crystalline cones had no optical power, an image would be formed at the rhabdom tips. If, however, the crystalline cone acts as a lens, then the system cannot be using simple focal optics.

The crystalline cones are so small ($2.5 \mu\text{m}$ wide proximally) that most measurement techniques, including interference

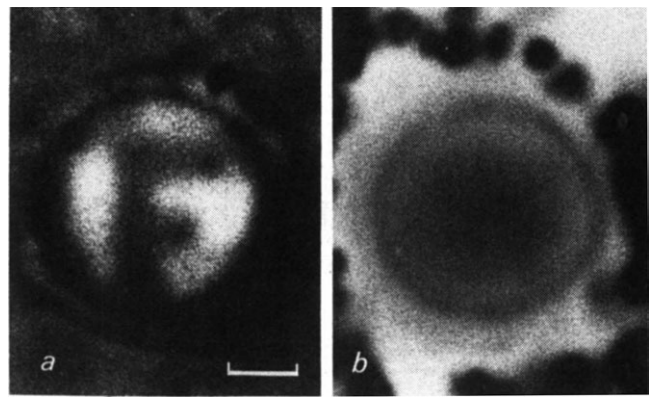


Fig. 1 Transverse sections of the proximal region of butterfly (*Heteronympha*) crystalline cones, cut in a cryotome after 1 h fixation. *a*, An image is formed of a distant letter 'F'. The inverted image is here re-inverted by the microscope. The focal length (in this case $5.1 \mu\text{m}$) was calculated from the relative size of the image. Sections with similar focal lengths were obtained from various different butterflies: *Junonia villida* (Nymphalidae), *Pieris rapae* (Pieridae), *Zizina labradus* (Lycaenidae) and *Papilio aegaeus* (Papilionidae). *b*, A protein gradient revealed by binding of toluidine blue (in *Heteronympha*). Scale bar, $1 \mu\text{m}$.

microscopy, are inadequate to resolve their optical structure. The method we finally used was as follows. Sections ($4\text{--}8 \mu\text{m}$) of stabilized material (1 h in 3% glutaraldehyde, 2% formaldehyde and 4% sucrose in 150 mM Na-cacodylate buffer) were cut in a cryotome at -14°C , then examined to determine whether they formed images (see Fig. 1*a*). Parallel-sided sections of the cones do indeed form inverted images and behave as powerful converging lenses. As judged from the unaffected pseudopupil, the optics were not damaged by the fixation. Staining of the sections with toluidine blue⁸ revealed a concentric protein gradient that is probably responsible for the focusing properties (Fig. 1*b*).

From the magnification of the images it was possible to work out the power ($1/f$) of sections taken at different levels along the cone. Figure 2*a* shows that most of the power lies in the proximal tip region; given this distribution, we were then able to trace rays through the cone by substituting each micrometre with a thin lens whose power is given by the ordinate in Fig. 2*a*. The results of this simulation are shown in Fig. 2*b, c*. Surprisingly, we found that the proximal region of the cone has exactly the right optical properties to convert the focused beam of light supplied by the cornea into a parallel beam whose diameter fits the rhabdom ($2.2 \mu\text{m}$). The weak middle region of the cone brings the converging beam to a focus $\sim 8 \mu\text{m}$ from the proximal tip of the cone, and in that $8 \mu\text{m}$ there is the equivalent of a lens powerful enough to re-collimate the focused light into a parallel beam directed down the rhabdom. It is noteworthy that this second lens is probably the most powerful known to man (0.2 megadioptries).

The optical properties of the ommatidium as a whole are accurately modelled by one spherical surface (cornea) and two thin lenses (cone) as in Fig. 3; it has the optical form of a telescope (in fact, properly known as a keplerian telescope with a Huygens eyepiece⁹). The angular magnification is 6.4, so that rays reaching the cornea at 1° to the optical axis emerge into the rhabdom at 6.4° (Fig. 2*c*). Note that the field of view of an ommatidium is now not determined by the rhabdom diameter, but by the maximum angle up to which the rhabdom will act as a light guide (in geometric optics this would be the complement of the critical angle). It also follows from the telescopic design that the rhabdom tip is imaged onto the cornea, magnified 9.1 times (the angular magnification multiplied by the refractive index inside the eye); this would mean that the $2.2 \mu\text{m}$ -wide rhabdom tip fills the central $20 \mu\text{m}$ of the $25 \mu\text{m}$ -wide facet, but in the domain of waveguide optics this cut-off will not be sharp.

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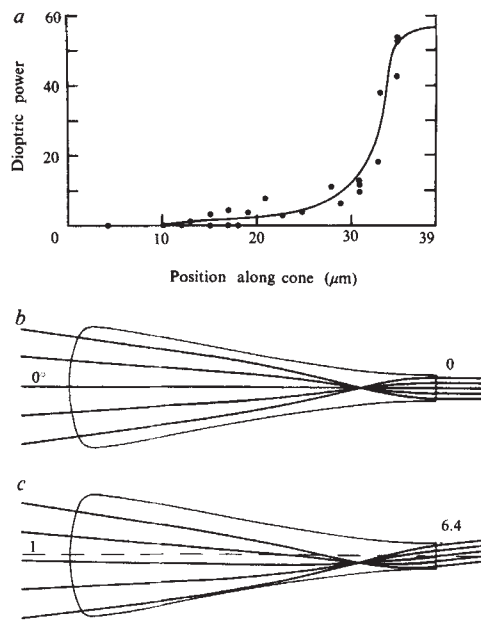


Fig. 2 Optical properties of the crystalline cone. *a*, A plot of the focusing (dioptric) power along the cone (in kilodioptries per μm). There is no power in the distal quarter but towards the proximal part the power rises monotonically, culminating as a powerful lens in the proximal tip. *b*, *c*, Rays are traced through a model cone where the focusing properties are simulated by a series of thin lenses (one per μm) with power as in *a*. The rays are parallel as they enter the cornea (not shown here) at 0° (*b*) and 1° (*c*), and emerge collimated from the cone at 0° and 6.4° , respectively.

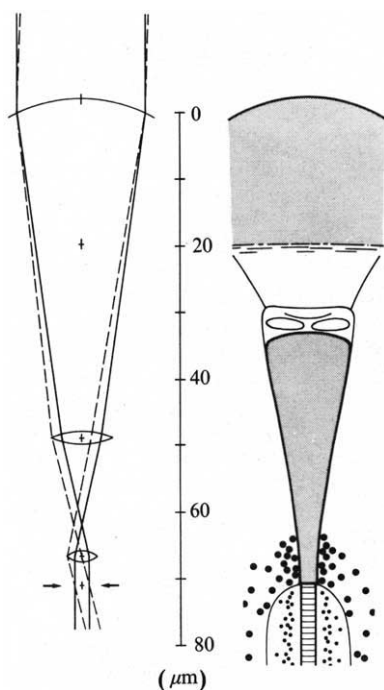


Fig. 3 The ommatidial optics is accurately modelled by one refracting surface ($f=49\mu\text{m}$) and two thin lenses ($f=37$ and $5\mu\text{m}$). The ray path is that of a telescope and is shown here by marginal rays entering at 0° (continuous) and 2° (dashed) to the optical axis. The rhabdom connects to the cone at the exit pupil (arrows) of the telescope. By following a pair comprising one dashed and one continuous ray, it can be seen that the rhabdom tip is imaged at the cornea. The corresponding anatomy is shown to the right: the crystalline cone is topped by its nuclei and separated from the cornea by a corneal process. The rhabdom (striped) is joined to the cone tip, and the surrounding regions are densely pigmented (represented by solid circles: large and small granules are in pigment cells and retinula cells, respectively).

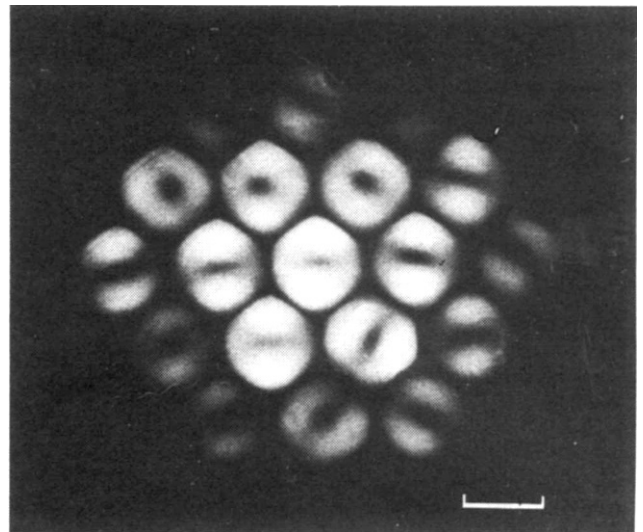


Fig. 4 Due to the reflecting tapetum, the eyes of many butterflies (here *Heteronympha*) shine brightly on incident axial illumination. The characteristic second waveguide mode, present at the rhabdom tips, is here seen imaged and magnified onto the corneal facets. Scale bar, $20\mu\text{m}$.

A valuable check on the magnification factor is given by the waveguide nature of the rhabdom—this provides us with luminous markers in the form of the second waveguide mode (Fig. 4). Most butterflies have a reflecting tapetum at the base of each rhabdom¹⁰⁻¹², and this makes the distal rhabdom tip shine brightly on incident axial illumination. Because of the cone-lens, the pattern of waveguide modes can thus be seen magnified on the cornea. The characteristically bi-lobed or 'doughnut'-shaped second waveguide mode is only present in some butterflies, apparently those with slightly wider rhabdoms, including *Heteronympha*. The first (or fundamental) mode, which has a simple, nearly gaussian intensity profile¹³, is always present but can be extinguished selectively by viewing the eye through crossed polarizers. The number of modes that are present and their size relative to the waveguide is a function only of the waveguide parameter¹⁴

$$V = \pi d(n_i^2 - n_o^2)^{1/2} / \lambda \quad (1)$$

where n_i and n_o are the refractive indices inside and outside the fibre, respectively, d is the fibre diameter, and λ is the wavelength of light. The second mode is only present when $V > 2.4$ (cut-off), and its high relative intensity in *Heteronympha* indicates that V is not close to cut-off. For values of V not immediately above cut-off, the separation of the two lobes will remain fairly constant at ~ 0.65 of the waveguide diameter¹⁵. Direct measurements gave a lobe separation of 14.1 ± 0.04 ($\pm$ s.d.) μm , making the rhabdom image $21.7\mu\text{m}$ in diameter and thus the magnification 9.9, close to the results obtained by ray-tracing. (In this calculation we have ignored diffraction effects: these will tend to blur the images of the modes somewhat, but should have little effect on the separation of the two halves of the second mode.)

Our demonstration of afocal apposition optics in butterfly ommatidia provides an interesting relation between butterfly apposition eyes and moth superposition eyes: both are afocal, but in the superposition eyes of moths and nocturnal beetles the angular magnification is low (1-2)¹⁶ and a real image is formed across a clear zone. In butterflies the angular magnification is much higher (here 6.4) and there is no clear zone to allow the formation of superposition images. It is easy to envisage a gradual transition from butterfly to moth eye or vice versa and, as recently reported for crustaceans⁷, insects now have a possible evolutionary link between apposition and superposition. However, we instead face the problem of transforming an ordinary focal apposition eye (as in bees) to an afocal one.

The final solution must await a detailed physical model comparing the relative merits of focal and afocal optics in apposition eyes.

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Differential responses to male song repertoires in female songbirds implanted with oestradiol

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Considerable debate surrounds the functional and evolutionary significance of the complex song repertoires found in male passerine birds¹⁻³. A comparative study on European warblers of the genus *Acrocephalus*⁴ has suggested that sexual selection is the driving force behind the evolution of the extraordinary song complexity in this group. One species in particular, the sedge warbler *Acrocephalus schoenobaenus*, sings long, variable, continuous songs constructed from a repertoire of discrete building blocks called syllables, revealed by sonographic analysis⁵. Although a strong inverse correlation between male repertoire size and pairing date was obtained from this wild population of sedge warblers, it was difficult to control adequately for confounding variables of male or territory quality which may also influence female choice at the time of pairing. It has been demonstrated recently that female songbirds implanted with oestradiol respond to playback of recorded song in laboratory conditions⁶⁻¹⁰; the resulting displays have been used as an index of response to a variety of natural and experimental songs. Here we have used both natural recordings from an earlier field study and experimental songs to demonstrate that implanted female sedge warblers respond significantly more to playback of higher repertoire sizes in standard laboratory conditions.

The first experiment was carried out in May 1983 on eight female sedge warblers. These were taken as nestlings from the Lake Neusiedl area of Austria in July 1982 and hand-raised in the laboratory. The birds were housed in groups in appropriate photoperiod conditions for trans-Saharan migrants. In April, the photoperiod was changed suddenly to local European conditions, and in early May each female received an oestradiol implant. Next, the birds were caged separately and housed in either a soundproof chamber or an isolated quiet room for 10 days before the experiments. On the day of the experiment, each bird in its holding cage was transferred to an experimental sound proof chamber fitted with a one-way observation window and

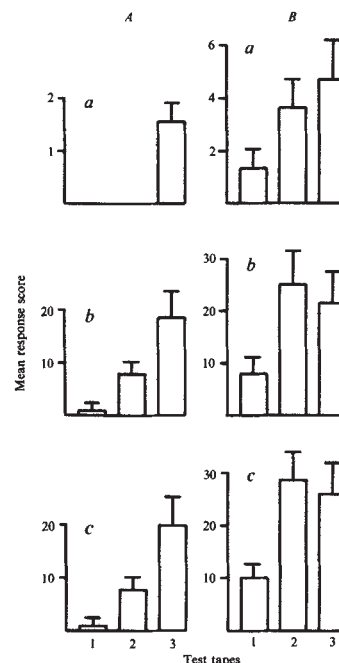


Fig. 1 Responses of eight oestradiol-implanted female sedge warblers to playback of recorded song from: A, three different species with different repertoire sizes; B, three male sedge warblers with different repertoire sizes. In A, test tapes consisted of 4 min of continuous song recorded from single males of: (1) blackbird *Turdus merula*, repertoire size 115; (2) reed warbler *Acrocephalus scirpaceus*, 80; (3) sedge warbler *Acrocephalus schoenobaenus*, 20. Responses measured were a, the number of wing vibration bouts; b, the number of horizontal crouches; c, the sum of these to give an overall response score. In B, the test tapes consisted of 4 min of continuous song recorded from males in an English population with repertoire sizes of: (1) low, 14; (2) medium, 24; (3) high, 41. Responses measured were the same as for A. The results shown are mean and standard error for the group. Each female received an implant of crystalline 17 β -oestradiol (Sigma E8E-8875) packed into Dow Corning 602-235 silastic tubing of 1.47 mm i.d. and 1.9 mm o.d. The tubing was sealed at both ends and implanted dorsally under the skin of the back where it could be seen and removed for later analysis.

a speaker. Each bird was exposed to 4 min of continuous song from males of three different species, in random order and separated by intervals of 4 min silence. The sound pressure level was standardized at 60-65 dB at a distance of 0.5 m from the speaker. Responding females became active and moved frequently into a horizontal crouching posture, occasionally vibrating their wings. Both these movements were counted separately and also totalled to give an overall response score.

Scores for the different song presentations have been compared and tested for significance using the Wilcoxon matched-pairs signed-ranks test, having first confirmed overall heterogeneity with Friedman's analysis of variance. Only sedge warbler song resulted in wing vibration, and the difference was found to be significant ($P < 0.025$) when compared with both reed warbler and blackbird (Fig. 1A). Although some crouching occurred to both reed warbler and blackbird song, the difference between these and the much larger response to sedge warbler song was highly significant ($P < 0.005$) for crouching and the total display score (Fig. 1A). The negligible baseline response to blackbird song is nevertheless an important control, as the repertoire size was nearly six times greater than that of the sedge warbler. The reed warbler had a repertoire size four times greater, and the more appreciable response obtained may in part be due to some similarities in song structure between congeneric species. Young hand-raised birds may also be expected to show rather less discrimination than wild adults, although the clear differences obtained suggest that a strong genetic preference for conspecific song structure is already established.

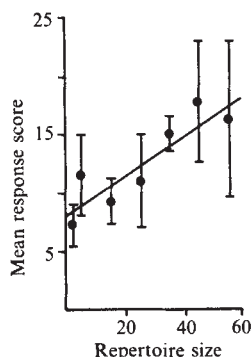


Fig. 2 The relationship between the mean response of five oestradiol-implanted female sedge warblers and the repertoire size of experimental male songs. The response score was obtained in the same way as for the first two experiments and is plotted as mean and standard error for the group. The correlation, however, is based on the original 35 data points ($r=0.342$, $P<0.05$). An independent correlation was also obtained between mean number of horizontal crouches and repertoire size ($r=0.335$, $P<0.05$). Wing vibration bouts accounted for only 10.9% of the total response, and so there were insufficient data for rigorous independent correlation analysis. However, the trend was in the same direction and, when included within the total response score, results in the slightly increased value of r . The experimental tapes were constructed from a recording of one male sedge warbler at Lake Neusiedl, Austria, by Professor A. Jilka, to whom we are most grateful. Seven tapes were made containing 4 min of continuous song from this individual, but with gradually increasing syllable repertoires of 2, 5, 15, 25, 35, 45 and 55. As the tapes were constructed from the same individual, singing rate and the total number of syllables presented remained virtually constant. The natural temporal patterning and sequencing of syllables was also maintained, and the bird merely cycled through smaller or larger repertoires of syllable types in a normal fashion.

The next experiment involved the same group of birds a few days later, and was designed to detect any preferences for conspecific males with larger repertoire sizes. Recordings of three sedge warbler males with different repertoire sizes were used to prepare 4-min tapes of continuous song as before. These were the two extremes and a middle range taken from the wild English population studied earlier. To avoid habituation to conspecific songs, the experimental design was modified so that although tapes were presented in random order only one presentation was made per day to each bird. We found that, in all three response categories, the male with the low repertoire size elicited significantly less response than the males with the medium or high repertoire ($P<0.01$ or $P<0.025$) (Fig. 1B). However, no significant differences could be found between the medium and high repertoire males. Although every attempt was made to hold constant all other variables except repertoire size, there were a number of inevitable differences between the three individuals. For example, the male with the medium repertoire sang much faster than the other two, and therefore presented the females with more syllables per unit time during the 4 min. It is likely that his faster singing rate compensated for his lower repertoire and thus abolished any differential response to the medium and high repertoire birds.

We therefore designed a final experiment in which all variables (including singing rate) were held constant, but repertoire size over a reasonable range was varied, to test whether females did in fact respond more to higher repertoires. We used one male and constructed a series of experimental tapes with gradually increasing repertoire size. The experiment was performed on a new group of five female sedge warblers taken as free-flying juveniles in the Neusiedl area in August 1983. The birds were housed and prepared as in the previous experiments and exposed to 4 min of continuous experimental song in random order on each day during May 1984. The results demonstrate a significant

correlation between repertoire size and response score (Fig. 2). We conclude that by increasing repertoire size a male sedge warbler also increases his probability of influencing female behaviour at the crucial time of female choice and pairing. These experiments support the view that sexual selection has an important role in the evolution of repertoire size and song complexity in some male birds.

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Plant-inducible virulence promoter of the *Agrobacterium tumefaciens* Ti plasmid

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Agrobacterium tumefaciens is the causative agent of crown gall, a plant tumour that can arise on most species of dicotyledonous plants. The tumour-inducing capacity of the bacterium requires the presence of a large plasmid, designated the Ti plasmid^{1,2}, which itself contains two regions essential for tumour formation—the T(umour)-region and the Vir(ulence)-region³. The T-region is transferred to plant cells by an unknown mechanism, and becomes stably integrated into the plant genome⁴⁻⁹. The Vir-region has been identified by transposon mutagenesis¹⁰⁻¹⁶, but the DNA of this region has never been detected in tumour lines^{4,5}. However, *trans*-complementation of Vir mutants¹⁷ indicates that genes of the Vir-region are functional in the bacterium. Moreover, the Vir- and T-regions can be physically separated in *A. tumefaciens* without loss of tumour-inducing capacity^{18,19}. Seven loci, designated *virA-F* and *virO* (refs 17, 20-22), have been identified in the Vir-region of the octopine Ti plasmid, but their functions are unknown. As *virC* mutants in the octopine-type plasmid pTiB6 are invariably avirulent in tests on various plant species¹⁷, this gene seems to be essential for virulence and we are studying it in detail. We report here that the promoter of *virC* shows no detectable activity in *A. tumefaciens* and *Escherichia coli* K-12 grown in standard medium, but that its activity is induced by a plant product.

To investigate the promoter activity of the *virC* gene, we constructed plasmid pMP30 (Fig. 1), which contains the complete *lacZ* gene of *E. coli* coding for β -galactosidase (EC 3.2.1.23), including the translational start signals, but lacks the *lacP-lacO* regulatory sequences. *E. coli* strains harbouring this plasmid show no detectable β -galactosidase activity, indicating the absence of readthrough events from plasmid promoters. Fragments of interest can be screened for promoter activity by cloning them in the unique *HindIII* site upstream of *lacZ*.

A 2.6-kilobase (kb) *HindIII* fragment containing the promoter region and part of the structural gene of *virC* was cloned in pMP30. Figure 1 shows the orientation of this fragment in

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Fig. 1 Construction of plasmids. The broad open bar indicates the *lac* genes. The solid bars indicate DNA derived from the *Vir*-region. The *Bam*HI-*Hind*III fragment of bacteriophage Mu d (*Ap*^r, *lac*) (refs 30, 31) containing the *lac* genes *Z*, *Y* and *A* of *E. coli* was cloned into pK04 (ref. 32). pRAL3551 contains the *Pst*I-O fragment from the *Vir*-region¹⁷. A 2.6-kb *Hind*III fragment was isolated from this plasmid, containing parts of *virO*, the promoter of *virC* and part of the structural gene of *virC*, but not the transcriptional stop of *virC* (ref. 17). This *Hind*III fragment was cloned in pMP30, resulting in pMP31. pMP31 was transformed into *E. coli* KMBL1164 (R772) and crossed with *E. coli* KMBL1224 (*Str*^r) with a frequency of 10⁻⁶. Sixteen transconjugants were crossed with *A. tumefaciens* LBA1010 Rif^r, Ti⁺ using Rif^r, Km^r, Ap^r selection in order to select for stable R772::pMP31 co-integrates. As pMP31—a pBR322 derivative—is not maintained in *A. tumefaciens*^{15,18}, Ap^r transconjugants should carry a stable co-integrate. A backcross to *E. coli* KMBL1164 confirmed this (100% co-transfer of the Ap^r and Km^r markers). One isolate, the R772::pMP31 co-integrate pMP33, was used to construct *A. tumefaciens* LBA288 (Rif^r, Ti⁻). The co-integrate R772::pMP30 (pMP32) was constructed in an analogous way. The insert shows the *Hind*III *virC* fragment present in pMP31 with the direction of transcription. Restriction endonuclease sites are indicated as follows: H, *Hind*III; B, *Bam*HI; S, *Sma*; P, *Pst*I; E, *Eco*RI; p.r. is the promoter region of *virC*; o.r.f. is the open reading frame of *virC*.

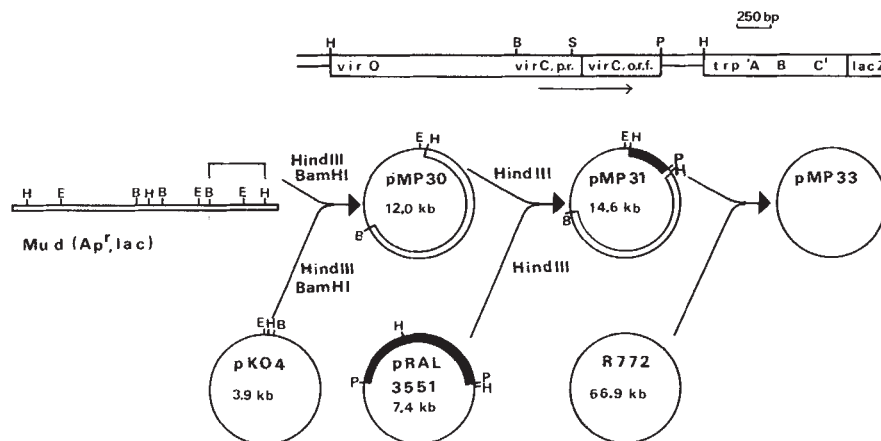


Table 1 Induction of β -galactosidase by *virC*-containing plasmids in the presence of plant exudates

Source of exudate	Bacterial strain/plasmid				No bacteria
	<i>E. coli</i> KMBL1164 pMP31	<i>E. coli</i> KMBL1164 pMP30	<i>A. tumefaciens</i> LBA1010, LBA88 pMP33	<i>A. tumefaciens</i> LBA1010, LBA88 pMP32	
<i>Pisum sativum</i> 'Finale' (Leguminosae)	++	—	+	±	—
<i>Vicia hirsuta</i> (Leguminosae)	++	—	ND	ND	—
<i>Daucus carota</i> , root culture (Umbelliferae)	++	—	+	±	—
<i>Nicotiana plumbaginifolia</i> , root culture (Solanaceae)	++	—	ND	ND	—
<i>Allium cepa</i> 'Rijnsburger' (Liliaceae)	++	—	+	±	—
<i>Zea mays</i> 'Zoete Bantammer' (Gramineae)	—	—	+ / ±	±	—
Minimal medium	—	—	±	±	—

Seeds of *P. sativum*, *V. hirsuta*, *A. cepa* and *Z. mays* were sterilized²⁷ and grown axenically for 4–8 days at 20 °C in the dark. Axenic root cultures were derived from *Agrobacterium rhizogenes* (hairy root disease)-infected plants of *N. plumbaginifolia* and from infected carrot disks (*D. carota*). Exudates were prepared by first washing the seedlings or root cultures in minimal medium for *E. coli*²⁸ or *A. tumefaciens*²⁹ for 5 min, followed by soaking for 4–5 h in the same medium. Exudates of *V. hirsuta* and *A. cepa* were prepared with 1 seedling per ml medium; exudates of *P. sativum* and *Z. mays* with 1 seedling per 3 ml medium. Exudates of root cultures were prepared with 0.1 g roots per ml medium. To eliminate plant fragments containing β -galactosidase activity, exudates were filter-sterilized before use. The pH of the medium did not change during incubation with the plant material. The sterilized exudates were inoculated with 5×10^7 bacteria ml⁻¹ and incubated for 16 h at 29 °C in the presence of 120 μ g ml⁻¹ X-gal. The bacterial concentration after incubation was $\sim 2 \times 10^8$ ml⁻¹. In a control experiment, lysates of *E. coli* (5×10^7 bacteria ml⁻¹) were tested for β -galactosidase activity with *O*-nitrophenyl- β -D-galactopyranoside (ONPG). A lysate of *E. coli* (pMP31) incubated with the exudate of *P. sativum* showed an A_{420} of 0.1–0.15 after 16 h of incubation at 37 °C with ONPG. Lysates of *E. coli* (pMP31) incubated in minimal medium and lysates of *E. coli* (pMP30) incubated in exudate showed an A_{420} of <0.001 in the same conditions. The intensity of the blue colour after reaction with X-gal was estimated and was correlated with the quantitative X-gal assay as described in Fig. 2 legend. ++, Deep blue colour ($A_{620} \geq 0.4$); +, blue colour ($A_{620} \geq 0.05$); ±, supernatant colourless ($A_{620} < 0.01$) and bacterial pellet very faint blue; —, no blue colour detectable; ND, not determined. *E. coli* KMBL1164 is F⁻ *del*(*lac pro*) thi; *A. tumefaciens* LBA1010 is Rif^r, Ti⁺; LBA288 is Rif^r, Ti⁻.

pMP31. It is possible that the *virO* promoter is also present in the cloned fragment, as the position of this promoter is unknown, but transcription from the *virO* promoter over the *virC* promoter into the *lacZ* gene seems unlikely. The direction of transcription of *virC*¹⁷ suggests that any β -galactosidase induction from pMP31 is due to the *virC* promoter.

Plasmid pMP31 in an *E. coli del*(*lac*) background showed a very weak but significant β -galactosidase activity after incubation for at least 60 h on indicator plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). No β -galactosidase activity was detected after growth for 48 h in minimal medium. pMP30, a pBR322 derivative, cannot be maintained in *A. tumefaciens*. In order to transfer and maintain pMP30 and pMP31, co-integrates with the broad host range plasmid R772 (ref. 23) were selected and crossed to *A. tumefaciens* strains LBA1010 (Rif^r, Ti⁺) and LBA288 (Rif^r, Ti⁻) (co-integrates pMP32 and pMP33, respectively). Neither co-integrate induced β -galactosidase activity in *A. tumefaciens*.

We tested whether *virC* induction was caused by the presence of plant factors (Table 1). A strong increase in *virC* promoter activity was detected in both *E. coli* and *A. tumefaciens* in the presence of exudates from a wide range of dicotyledonous varieties. The response in *E. coli* was stronger than that observed for *A. tumefaciens*, probably because the copy number of the expression vector is high (~ 40) in *E. coli* and low (~ 4) in *A. tumefaciens*. The response of the *virC* promoter on exudates of monocotyledonous plants varied with the species used. Exudates of *Allium cepa* were as effective as dicotyledonous exudates in stimulating the *virC* promoter, whereas exudates of *Zea mays* showed little stimulatory activity (Table 1). β -Galactosidase activity was undetectable in most exudates but those which did contain some activity were not used for promoter-activating studies. As the cells of the plants used contain substantial β -galactosidase activity, homogenates of plant material were not tested. No β -galactosidase activity was detected in control *E. coli* containing pMP30 or pMP32. The activity in *A.*

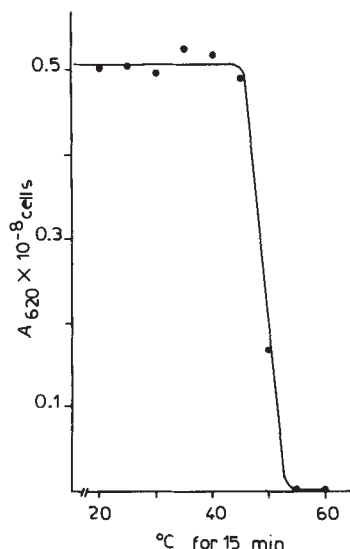


Fig. 2 Thermo-inactivation curve of *Pisum sativum* exudate. Exudate of 8-day-old seedlings was filter-sterilized and kept for 15 min at the indicated temperatures. The tubes were transferred to 29 °C and *E. coli* KMBL1164 (pMP31) and X-gal were added. After reaction, the bacteria were lysed with lysozyme, 400 µg ml⁻¹ for 10 min at 20 °C, followed by 20 mM NaOH, 0.1% SDS for 5 min at 100 °C. After centrifugation for 5 min at 15,000 r.p.m., the extinction of the coloured supernatant was determined at 620 nm. The thermo-inactivation was used in a control experiment to determine whether the induction factor works on the bacteria *per se* or on a component of the bacterial culture medium. Root exudate of *P. sativum* (filter-sterilized) and filter-sterilized culture medium of *E. coli* pMP31 were mixed 1:1 and incubated in the presence of X-gal for 3 h. No blue colour developed, indicating that bacteria are needed for β -galactosidase induction. Subsequently, the mixture was heat-inactivated for 15 min at 55 °C, cooled at 29 °C and *E. coli* pMP31 added. The bacteria were incubated for 16 h at 29 °C. No blue colour developed, indicating that the inducer works directly on the bacteria and not on a component of the bacterial medium.

tumefaciens (pMP32) was not above the very low background due to *A. tumefaciens* β -galactosidase itself. In conclusion, a factor present in plant exudates strongly activates the promoter of the *A. tumefaciens* *virC* gene.

In an attempt to identify factors that could be responsible for the promoter-activating property of exudates, several compounds which could be excreted by plants and could thus be present in exudates were tested with *E. coli* KMB1164 (pMP31). None of the compounds tested—amino acids (casamino acids 0.3%), pantothenate, nicotinic acid, *p*-aminobenzoic acid, pyridoxine, thiamine (400 ng ml⁻¹ each), arabinose, lactose, galactose, sorbitol, mannitol, xylose, melibiose, cellobiose myo-inositol (6.6 mg ml⁻¹ each) and the plant growth substances kinetin (6.5 µg ml⁻¹) and naphthalene acetic acid (6.5 µg ml⁻¹)—showed promoter-inducing activity.

Control experiments (Fig. 2 legend) showed that the inducer works on the bacteria *per se* and not on a component of the bacterial culture medium. The exudate is heat-labile, showing a sharp decline in activity on incubation at 46 °C or higher and becoming completely inactivated after 15 min at 55 °C (Fig. 2). The inducing activity is not affected by treatment with RNase I, DNase, phospholipase A₂ or phospholipase D, but was abolished completely on incubation with 20 µg ml⁻¹ pronase or trypsin. Control experiments with *E. coli* CSH23-F^{lac} *del*(*lac pro*) in the presence of the *lac* inducer isopropyl- β -D-thiogalactopyranoside showed that treatment of the exudate with the enzymes did not affect the viability or β -galactosidase-inducing capacity of *E. coli*.

The exudate factor was retained on equilibrium dialysis using membranes (Spectrapore) with an indicated cut-off for globular

molecules of relative molecular mass (M_r) > 7,000. Thus, the inducer is probably proteinaceous in nature, with an estimated M_r > 7,000. Further purification and characterization of the inducer is under way in our laboratory.

Our results demonstrate for the first time the existence of a plant product that regulates the virulence of a plant pathogenic bacterium. The plant product does not require the presence of the bacterium for its appearance nor to inflict severe wounding, which is known to be a prerequisite for tumour induction. We believe that the mechanism of promoter induction is indirect because the inducer is too large to pass through the pores of the outer membrane of *E. coli*, which in general are not permeable to molecules of M_r > 700 d (for a recent review see ref. 24). The promoter described here could be of practical use in the construction of bacterial strains meant to produce substances only on contact with the plant, for example siderophores^{25,26}. The method described can be applied to the detection of other plant-regulated promoters in bacteria that interact with plants.

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Evolutionary origin of a calcium-dependent protease by fusion of genes for a thiol protease and a calcium-binding protein?

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Calcium-dependent protease (calcium protease) is apparently involved in a variety of cellular processes^{1–3}. Here we have attempted to clarify the role and regulatory mechanism of calcium protease by analysing its structure. The complete primary structure of calcium protease (relative molecular mass (M_r) 80,000 (80K), 705 amino acids) was deduced from the nucleotide sequence of

cloned complementary DNA. The protein contains four distinct domains, and we have observed a marked similarity between the second and fourth domains and the papain-like thiol proteases and calmodulin-like calcium-binding proteins, respectively. This finding suggests that calcium protease arose from the fusion of genes for proteins of completely different function and evolutionary origin. Further, it provides functional insight into cellular regulatory mechanisms mediated by Ca^{2+} through calcium-binding proteins.

The nucleotide sequence of calcium protease messenger RNA (Fig. 2) was deduced from the sequences of two recombinant plasmids, pCP521 (cDNA insert, 2,219 base pairs, bp) and pCP1 (cDNA insert, 2,024 bp). The two cDNAs span 3,368 bp and the nucleotide sequence in their overlapping region is identical (875 bp). DNA sequence analyses revealed total compatibility with the partial amino acid sequence of calcium (or structure of calcium) protease determined separately. Only one open frame can encode a protein of M_r 80K, and mRNA for calcium protease was detected as a single band of 3.5 kilobases (kb) by blot hybridization of poly(A) RNA from various chicken tissues (Fig. 1a). As the cDNA was 3.4 kb long, the cloned cDNA contained almost all of the mRNA sequence, but not the complete 5'-flanking sequence.

Southern analysis⁴ of total genomic DNA suggested the existence of an essentially unique gene for the calcium protease mRNA (Fig. 1b). By S_1 mapping experiments⁵, the 5' end of the exon containing the 5' region of the deduced mRNA sequence was localized at around -37 in the cloned genomic DNA sequence, allowing the 5' sequence of the mRNA to be determined. We concluded that the AUG at position 1 is the starting signal for protein synthesis (Fig. 2).

The mRNA for calcium protease encodes a protein of 705 amino acids and M_r 80,350. The N-terminus of calcium protease is blocked. N-terminal sequence analysis of the autodigestion product (M_r 78K)¹⁻³ gave a sequence identical to residues 18-26; a blocked peptide corresponding to residues 2-17 was also isolated from the digestion mixture. Thus, the N-terminus of the mature calcium protease is methionine at position 2. Carboxypeptidase Y digestion of calcium protease quantitatively liberated amino acids that were compatible with the C-terminal sequence from 692 to 705, indicating that Gly 705 is the C-terminus of the mature calcium protease. These results imply that calcium protease is not processed proteolytically.

Two types of calcium protease, one with low (m type) and one with high (μ type) affinity for Ca^{2+} , are generally found in mammalian tissues¹⁻³; chicken tissues, however, contain only m type calcium protease⁶. The enzyme comprises two components of M_r 80K and 30K¹⁻³. The 80K protein shows the same catalytic and regulatory properties as the native enzyme^{6,7}, but the role of the 30K protein remains uncertain. The 80K protein should possess at least two properties, proteolytic activity and calcium-binding activity.

Calcium protease is classified as a thiol protease⁸. Comparison of the amino acid sequence around the active site, Cys 108, with those for other thiol proteases (for example, Cys 29 in cathepsin B) revealed significant homology in a much longer sequence than previously noted (Fig. 3a)⁸. Another region around His 265 is also homologous to the sequences around the active-site His residue (His 197 in cathepsin B) of other thiol proteases. This strongly suggests that, as with other thiol proteases⁹, His 265 is essential for catalysis.

Chicken calcium protease has several calcium-binding sites, at least one of which has a low affinity for Ca^{2+} and one a high affinity (ref. 10). A typical structure, known to be a calcium-binding site, is the 'E-F hand' structure^{11,12}. All proteins characterized so far which contain high-affinity calcium-binding sites, for example, calmodulin, troponin C and myosin light chain, contain E-F hand structures, and constitute a protein superfamily with the same evolutionary origin^{13,14}. A search for such an E-F hand structure in the calcium protease sequence, using the method of Tufty and Kretsinger¹², revealed four candidates, at positions 589, 619, 654 and 684. Of these, those structures

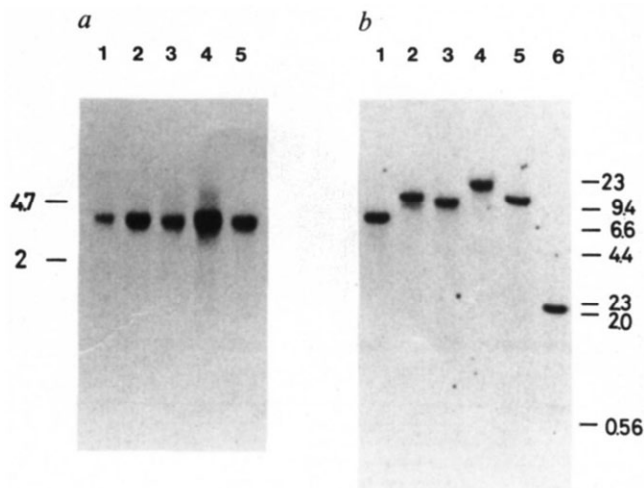


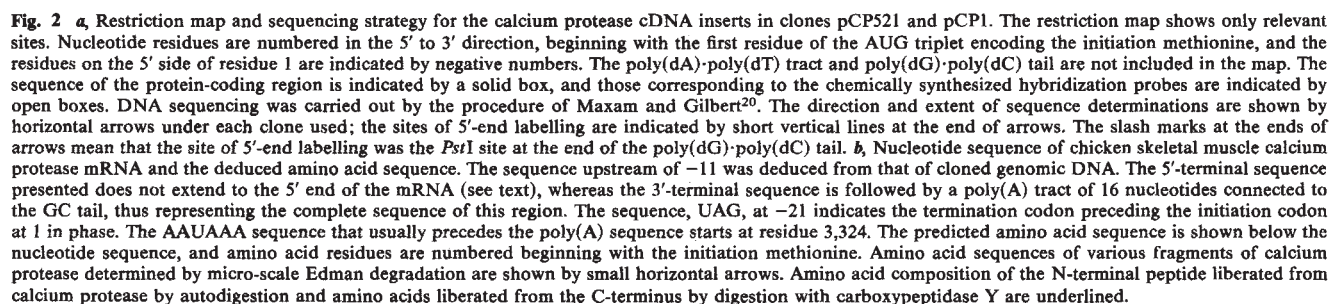
Fig. 1 a, Identification of calcium protease mRNA in various chicken tissues by blot hybridization using a cDNA for chicken calcium protease as a probe. Lanes: 1, brain; 2, spleen; 3, muscle; 4, lung; 5, kidney. The size marker was mouse ribosomal RNA. b, Identification of the calcium protease gene by blot hybridization analysis of chicken cellular DNA. Lanes: 1, *Eco*RI and *Hind*III; 2, *Hind*III; 3, *Hind*III and *Bam*HI; 4, *Bam*HI; 5, *Bam*HI and *Eco*RI; 6, *Pst*I. Size markers were λ *Hind*III digests. Numbers on the right and left-hand sides are size markers (kb).

Methods: a, Poly(A) RNA (5 μ g) was denatured with 2.2 M formaldehyde and 50% formamide, electrophoresed on 1% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose paper²². The hybridization probe used was a *Pst*I fragment (residues 31-1,128). b, Total chicken cellular DNA was digested with restriction enzymes, electrophoresed on a 0.8% agarose gel and subjected to blot hybridization analysis⁴ using a *Pst*I fragment (residues 31-1,128) as a probe.

starting at residues 589, 619 and 684 contain highly conserved sequences (33-50%) seen in 30 established calcium-binding sites, and consisting of 12 amino acids¹⁵ (typical examples are given in Fig. 3b). Their predicted secondary structure suggests that these potential calcium-binding sequences are located between two α -helical regions. These sites should therefore function as calcium-binding sites in calcium protease. All are found at the C-terminal region of calcium protease, and their spacing along the protease chain resembles that in other calcium-binding proteins (Figs 3b, 4).

We can now develop a schematic structure for calcium protease which is composed of four distinct domains: domain I, residues 1-80; domain II, residues 81-320; domain III, residues 321-560, and domain IV, residues 561-705 (Fig. 4). The function of domains II and IV was clarified using sequence homology and previously reported results, but the function of domains I and III remains unknown; perhaps they act as intramolecular regulators of the catalytic activity residing in domain II or as binding sites for the 30K protein, inhibitor protein¹⁻³ or activator protein¹⁻³.

Calcium is essential for the proteolytic activity of calcium protease; thus, the activity of domain II is suppressed—possibly by domains I and/or III—when it is incorporated in the complete calcium protease molecule. The role of the calcium-binding domain (domain IV) is to derepress the protease activity of domain II. In this respect, the role of domain IV is similar to that of calmodulin which, in the presence Ca^{2+} , activates (or derepresses) enzymes such as phosphodiesterase¹⁷ or phosphorylase b kinase¹⁸ by means of protein-protein interaction. However, there is an important difference between the regulation of enzyme activity by the calcium-binding domain of calcium protease and by calmodulin. This is that the calcium protease calcium-binding domain (domain IV) is covalently linked to the target protease portion (domain II) through domain III. This


$$3'-CT\overset{A}{\underset{G}{T}}ACCTNCG\overset{A}{\underset{C}{T}}CA-5',$$


synthesized and used as two pools containing A/T or G at the sixth position, designated as N, which correspond to the amino acid sequence at positions 99–103 (see *b*). Total RNA was extracted from chicken skeletal muscle²¹, and poly(A) RNA was isolated by oligo(dT)-cellulose chromatography²². Poly(A) RNA was fractionated by sucrose density centrifugation and assayed by translation in a rabbit reticulocyte lysate system²². Translation products were analysed by SDS-gel electrophoresis after immunoprecipitation with rabbit antibody against chicken skeletal muscle calcium protease. Fractions giving an 80K protein band were pooled and used as a template for cDNA synthesis. Double-stranded cDNA was prepared by standard methods using poly(A) RNA from the 25–26S fraction²². cDNA >1.5 kb was isolated by agarose gel electrophoresis and annealed with plasmid pBR322 by the GC-tailing procedure²². *Escherichia coli* χ 1776 was used for transformation, and tetracycline-resistant transformants were screened *in situ* by hybridization²² at 40 °C or at 37 °C with the synthetic oligodeoxyribonucleotide mixtures no. 20 or no. 30, which were labelled with ³²P at the 5' end²⁰, respectively. One recombinant clone (pCP521) which hybridized with both of the two probes was isolated from 1 × 10⁴ transformants. Another clone (pCP1) was isolated from the same cDNA library by hybridization with a mixture of two *Pst*I fragments (31–1,128 and 1,128-end of the GC stretch of the 3' end) of the pCP521 insert which was labelled by nick-translation. Three hybridization-positive transformants were isolated; one of them (pCP1) was subjected to DNA sequencing in addition to pCP521. Both DNA strands were sequenced and all the restriction sites used for sequencing were read through by sequencing begun at other restriction sites. *h*, A chicken genomic DNA clone, λ CP115, was isolated from the chicken genomic DNA library provided by Dr J. D. Engel. To determine the 5'-flanking sequence of calcium protease mRNA, which was not cloned into the cDNA recombinant pCP521, the genomic DNA was analysed by sequencing and S₁ nuclease mapping⁵. A single-stranded DNA probe spanning from –354 (*Eco*RI site) to 39 (*Ava*II site) 5'-end-labelled at the *Ava*II site was hybridized with poly(A) RNA from chicken skeletal muscle and the S₁-resistant DNA was analysed.

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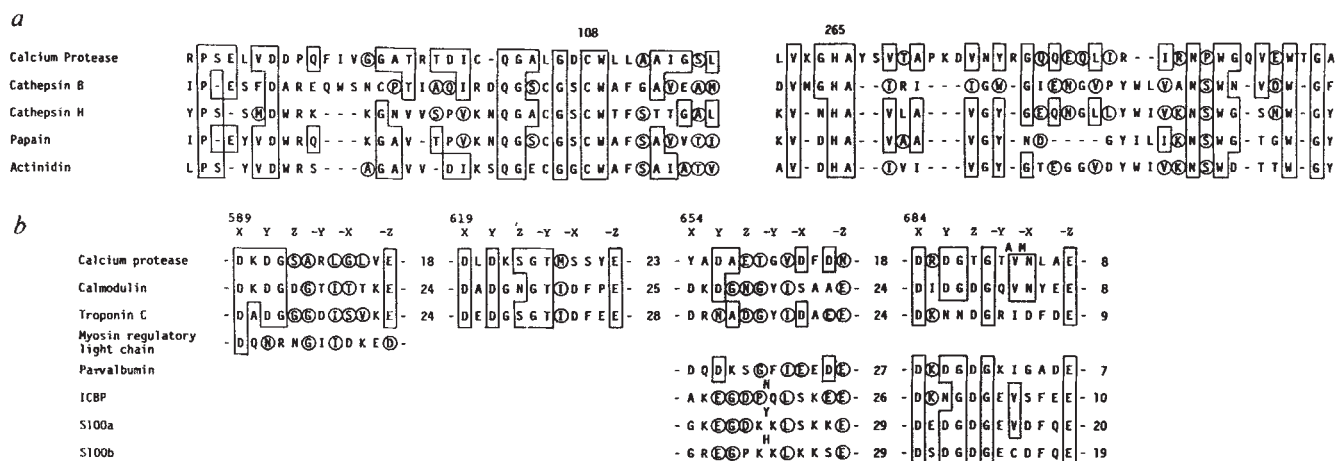


Fig. 3 *a*, Amino acid sequences of subdomains containing the active-site Cys and His residues in calcium protease. Corresponding sequences of thiol proteases⁹ are aligned for comparison. The one-letter amino acid notation is used. Residues identical to those in calcium protease are boxed and circled residues represent favoured amino acid substitutions. Favoured amino acid substitutions are defined as described previously²³. Numbers above the sequence correspond to the sequences of calcium protease (see Fig. 2*b*). Gaps (-) are inserted to achieve maximum homology. *b*, Amino acid sequences of four predicted calcium-binding sites. Corresponding sequences of calcium-binding proteins are aligned for comparison. Residues corresponding to the calcium-binding loop region are shown, where X, Y, Z, -Y, -X and -Z represent the calcium coordinating positions¹². Numbers shown at the top of the sequences indicate the residue numbers of calcium protease at position X. Numbers shown between amino acid sequences represent those of amino acid residues between calcium-binding loops. Numbers shown in the right-hand row indicate residue numbers present up to the C-termini following the calcium-binding loop. Proteins used; calmodulin (human), troponin C (rabbit), myosin regulatory light chain (rabbit), parvalbumin (carp), ICBP (bovine intestinal calcium-binding protein) and S100a, b (α and β -subunits of bovine brain S100 proteins).

Methods: *b*, E-F hand calcium-binding sites were searched for by the method of Tufty and Kretsinger¹² in the calcium protease sequence. The alignment for the test sequence, that is, the number of residues identical to the test sequence at 16 positions within and around the calcium-binding loops^{12,24}, was 11 for each of the four sequences. The second class of high-score sequences lie at positions 154, 396 and 545, with scores of 9. Scores of established calcium-binding sequences vary from 12 (domain III of carp and rabbit parvalbumin) to 16 (domain II of rabbit troponin C). The scores of potential calcium-binding sequences in rabbit myosin regulatory light chain (II, III and IV) were 10, 10 and 11, respectively.

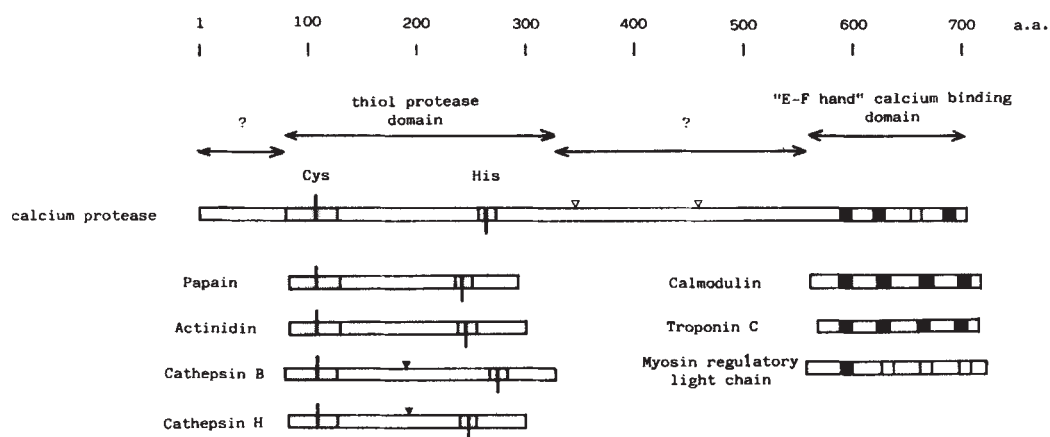


Fig. 4 Schematic representation of the structure of calcium protease and comparison with thiol proteases and calcium-binding proteins. Amino acid sequences around the active-site Cys and His residues shown in Fig. 3*a* are indicated by shaded boxes. Upward- and downward-projecting lines show the active-site Cys and His residues. Predicted calcium-binding sites composed of 12 amino acid residues are represented as solid boxes, and potential sequences for calcium-binding sites are shown as open boxes. Δ , The attachment sites of carbohydrate⁹; ∇ , in calcium protease indicates the potential attachment site of carbohydrate. Numbers indicate the positions of amino acid residues in calcium protease (see Fig. 2).

the chicken genomic DNA library. This work was supported in part by research grants from the Ministry of Education, Science and Culture, and by a grant-in-aid of new drug development from the Ministry of Health and Welfare, Japan.

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Holland's Earth

James E. Lovelock

The Chemical Evolution of the Atmosphere and Oceans.

By Heinrich D. Holland.

Princeton University Press: 1984. Pp.582. Hbk \$97.50, £80.70; pbk \$24.50, £20.30.

THERE is an almost sensual pleasure to be had from collecting small rare objects, such as stamps, books or rocks. Some modern books on natural history can delight the reader in the same way, but it is unusual for a textbook to elicit this feeling. *The Chemical Evolution of the Atmosphere and Oceans* is an exception.

Professor Holland's view of the evolution of the Earth — where the constancy of climate and composition over geological time is a straightforward consequence of the working of chemical and physical forces — differs sharply from mine that sees in the constancy a homeostasis maintained for and by the biota. That this difference is mutual is noted courteously but firmly in his closing pages. In no way does this difference of interpretation alter my pleasure from reading the book, nor my admiration of its authoritative excellence — science can benefit from the personal dialectical approach and guesses are needed to start the iterations that may converge towards the unattainable goal of truth. The book is enhanced by clear and comprehensible graphs and tables, and at times reads more like the report of a research project than a textbook.

It begins with four chapters about the origins of the Earth and on what is known of the Hadean. It is easy to forget that this turbulent period occupied nearly one-quarter of the Earth's history, whereas human beings have occupied only the past thousandth of it. Models of the Hadean are thin and rather speculative but here are displayed the few solid facts that are available. It is a source of wonder that one of the most certain of these is the earliest; namely, that much of the material of the Earth came from a supernova explosion 4.55 Gyr ago. The evidence from the distribution of radioactive and stable isotopes, the fall-out of that vast initial nuclear explosion, is used extensively throughout the book. Just as loud and angry voices carry further than quiet conversation, there is a tendency to concentrate on events that are violent and stormy rather than on the calmer periods in between; yet it could have been during a calm spell at the end of the Hadean that life assembled itself. Essential for theories of the origin of life is information on the structure and composition of the prebiotic atmosphere and oceans. It seems that the new orthodoxy is still with us and that the atmosphere at the end of the Hadean was dominated by CO₂ and neutral to slightly reducing. The oceans by contrast were buffered at a low

redox potential by an abundance of soluble electron-rich species of elements such as iron and sulphur.

As the book moves on into the Archean, the emergence of life is noted but curiously kept at low key as if the author considered it to be impolite or improper to discuss biological matters too deeply in a book on chemical evolution. Perhaps, though, the reluctance to become involved with the fashionable science of biogeochemistry is wise. There have been numerous attempts

IMAGE
UNAVAILABLE FOR
COPYRIGHT
REASONS

Atmospheric input — Krakatoa rumbles on, one hundred years after the original eruption.

to put the biota in a box and assign to it inputs and outputs, an operation analogous to that of making a materials and energy balance of a living animal. Something may be learnt from these exercises but they can offer nothing to explain the potential of living systems for homeostasis nor can they predict the course of their evolution. An interdisciplinary approach to earth science is more in need of contributions from geophysics than from biogeochemistry.

This beloved data-bank of a book that never considers things beyond the limits of decent speculation also never ceases to present its data in a way that tempts speculation far beyond those limits. To nowhere does this apply more than the chapter on terrestrial environments. The environmental factors that determined the speciation of uranium at the end of the Archean are discussed at length yet there is

no word about the strange affair of the natural nuclear reactors at what is now Oklo in Africa. There, 2.35 Gyr ago, uranium was sufficiently concentrated and purified to exceed the critical mass needed for a working nuclear reactor. Did the primitive artifice of dumb prokaryotic miners equal our proud science and technology? And what does the existence of that reactor then tell us of the atmosphere of those times?

The personal character of the book is strongest in the final chapter that deals with the Phanerozoic. The fascinating question of why the composition of the sea has stayed so constant is made significant by the skilful marshalling of the evidence. All is included, even the poetic names of those extraordinary crystalline rocks of the evaporites; such as polyhalite that bears no halogens or sylvite that does. From the constancy of the composition of sea water we move on to the constancy of the abundance of oxygen in the recent atmosphere. I could not help wondering here why the evidence of the presence of charcoal and of fossilized wood in sediments is not mentioned as setting quite sharp limits on the possible range of oxygen. If charcoal is a fossil indicator of fires, the fires can only have occurred when oxygen was within the range 15% to 25%.

The evolution of the Earth as a planet is vastly different from that of the other terrestrial planets. As Anderson has recently observed (*Science* 223, 347–355; 1984), plate tectonics are unique to the Earth and made possible only by the presence of limestone, which in turn is almost wholly a product of the biota. The geological community is only now beginning to take on the difficult problem of explaining the effects of the presence of the biota on the evolution of the Earth, and it will be some time before the biological presence is considered as a driver rather than a passenger. There are still formidable barriers to interdisciplinary thinking.

As a young scientist I was advised to read original papers rather than textbooks. Times have changed — nowadays too many published papers are either otiose or just plain wrong. How can those who seek information outside their speciality sift the good from the bad? This is where a book such as this is invaluable to a general scientific readership. It is well and clearly written, providing an authoritative summation of information about the Earth from the viewpoint of a geologist and a chemist, and also serves as a guide to the key original sources. But most of all it contains a constant supply of robust but finely tested facts and decent speculations that can be used for building images and models. It will be my companion for many years to come. □

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Building the modern main-frame

John Hendry

The Computer Comes of Age: The People, the Hardware, and the Software. By R. Moreau. Translated by Jack Howlett.

MIT Press: 1984. Pp.227.
\$19.95, £20.85.

THERE are remarkably few books on the recent history of computing. Accounts of the prehistory, from Babbage to the Second World War, abound, as do reminiscences of the pioneering wartime and immediate post-war projects. A start has even been made — for example by Nancy Stern in *From ENIAC to UNIVAC* — on a genuinely historical analysis of these projects. But there is still precious little on events since 1950, and virtually nothing in accessible book form.

The Computer Comes of Age, a translation of René Moreau's 1981 book *Ainsi Naquit l'Informatique* (French birth corresponding, apparently, to English adulthood!), is the first significant exception to this rule. The work is a chronicle rather than a history and the style of presentation, though an improvement on the French edition, does not make for easy reading. The report-like format, with short headed sections, many only a paragraph long, makes few concessions to narrative continuity. Indeed, as one advances through the book and the technological developments, all of which are explained for the benefit of the non-specialist, get more complex, one becomes increasingly aware of a similarity with computer training manuals. The focus is almost wholly on the technology, with nothing on the social or economic aspects of computer development, and because the work is based largely on "common knowledge" rather than historical research it is far from being authoritative. Finally, even Moreau closes his account with the arrival of the IBM series 360 computers in 1963, a recent event by normal historical standards, but a very distant one if one considers how much has happened since that time.

In short, then, this is not really a book for casual reading, for those interested more in the computer age than in the computers (and computing languages) themselves, or for those seeking a definitive history. Nonetheless, within its very reasonable limits it is a valuable work.

The strength of the book lies in its comprehensive coverage of the myriad of technical developments leading up to what was, in effect, the modern main-frame computer. From ferrite-core memories to front-end processors and from non-local concurrency to the characteristics of COBOL, pretty well every important

development of the period is covered, be it in hardware, software, peripherals or operating methods. Almost all the most interesting computers of the period are described, albeit briefly, with mention of their particular innovations and the degrees to which they incorporated emergent technologies. The often-neglected problems of operation and communication, and the development of programming languages, are also brought in.

It is, however, a pity that the laudable effort to make the subject accessible could

not have been accompanied by a similar effort to make it attractive; as it stands, the book is unlikely to appeal greatly to the computer-non-literate population for whom it was apparently intended. But the fact is that no one has yet done any better, or even anywhere near as well in tackling the subject. Until they do, René Moreau's book well deserves whatever success it may find. □

John Hendry is the ESRC Fellow in Business History at the London Business School, and is currently preparing a history of the British computer industry.

Principle patterns

Brian Goodwin

Pattern Formation: A Primer in Developmental Biology.

Edited by George M. Malacinski and Susan V. Bryant.

Macmillan, New York/Collier-Macmillan, London: 1984. Pp.626. \$58, £55.

GEORGE Malacinski and Susan Bryant have brought together an excellent collection of essays on pattern formation. No volume could be comprehensive and definitive in such a diverse and rapidly-moving field, but this one comes close. Not only is the range of organisms treated very extensive, from the ciliate protozoa and *Volvox* to higher plants and animals (though nothing on mammals!); but the conceptual scope is also broad, including work informed largely by classical embryological concepts, sophisticated analytical treatments by the model-builders, and detailed analyses of the cellular, genetic and molecular mechanisms involved in pattern formation. Few subjects can boast such an apposite pluralism, for the whole organism together with all its parts continue to exist as real structures in developmental biology. Dominant themes nevertheless come and go. *Pattern Formation* celebrates a period of research whose intellectual liveliness, balance between empirical discovery and conceptual interpretation, and sheer enjoyability owe much to the personality of one individual, Lewis Wolpert. It is a further tribute that this volume has within it not only material that derives directly from his work, but indications of what type of analytical framework may supersede it.

The book starts with summaries of discussions between Malacinski and some of the contributors, the first of whom states that Wolpert's theory of positional information is not a falsifiable theory, but a concept, a framework for the interpretation of phenomena. In his article with Stein, however, Wolpert says that "the concept of positional information was explicitly formulated by Driesch", arising from his observation that sea urchin blastomeres are

totipotent, their actual fates therefore depending upon their spatial relations to other cells in the embryo. This was a testable theory, and my understanding is that Wolpert takes the same view of his particular formulation of Driesch's proposition. Certainly, several contributors treat it as such and test it critically against empirical evidence. It is worth noting in passing that in converting Driesch's concept of relational spatial order into the notion of a coordinate system within the organism, Wolpert constructed a conceptual framework, via the notion of free and unconstrained interpretation of "positional values" by species-specific genetic programmes, which opened the door wide to the Darwinian principle that any organismic form is possible, natural selection then making its choice from the continuum. Driesch vehemently opposed this view, planting his banner firmly with the rational morphologists and "laws of form".

Accounts of the search for generative rules or laws operating during development occupy a significant part of this volume, as Susan Bryant observes in her introductory comments. This reflects an important transition in the evolution of positional information models from their early, virtually unconstrained form to ones embodying abstract generative rules such as those of the polar coordinate model (well represented in articles by Held and Bryant on *Drosophila*, and by French, Holder and Javio on insect, amphibian and chick limbs respectively). Perhaps the most significant consequence of this model is its demonstration that simple rules with empirical consequences can be formulated without reference to molecules, or even cells, capturing principles of spatial organization that extend across an impressive range of taxa. The ingenious application by Frankel of these principles to the explanation of supernumerary oral apparatuses with reversed symmetry in *Tetrahymena* reminds us that pattern formation is not, in fact, dependent upon cellular interactions. Thus we seem to catch a glimpse of universal principles, the general spatial properties of which are developed with characteristic incision by Winfree.

The richness of morphogenesis, analytically explored in "simple" forms such as *Volvox* (Sumper), *Dictyostelium*

(MacWilliams), *Hydra* (Bode and Bode), and in more complex organisms such as insects (Sander; Anderson and Nüsslein-Volhard), higher plants (Sachs, McDaniel), sharks (Reif), amphibians (Malacinski, Slack, Cooke, Stocum and Fallon) and chicks (Dhouailly), force an elaboration of concepts beyond either the original positional information theory or the polar coordinate model. Here we find a profusion of developmental mechanisms, some classical and some modern, all very clearly elaborated. Both Kauffman and Meinhardt achieve some success in accounting for certain aspects of these phenomena by the discovery of new properties of old models, and by adding dynamical features with switching logic and recursive properties to generate structures such as segments and compartments, and models of distal transformation in insects.

But for me the high point of the volume, whence one catches a glimpse of a way of putting together all that has come before but within a somewhat different scheme, arose from a certain reading of the chapters by Karlsson, Poethig and Nuccitelli — the first on *Drosophila*, the second on angiosperm leaf morphogenesis and the third on electrical fields in morphogenesis (plant and animal, unicellular and multicellular). The first two authors interpret pattern formation in terms of fields of mechanical force (mediated by the cytoskeleton) that both guide and are guided by patterns of cell division and differentiation in *Drosophila* imaginal discs and in leaf meristems; while the Nuccitelli paper on ionic currents indicates the dynamic boundary conditions for affecting the state of the cytoskeleton and cell metabolism.

Neither Karlsson nor Poethig sees evidence in their material for either a logical gene-regulated decision process resulting in spatial patterns, or for gradients or "coordinate-systems" that spatially control such decisions. Since Sydney Brenner, somewhat impishly, has recently said about the explanation of pattern formation by molecular mechanisms of gene control: "I doubt whether anyone believes that anymore", we can now feel free to seek alternatives. One possibility is to understand morphogenesis as a unitary process in which global mechanical fields (of strain, or strain-rate, for example), acting through and on both the cytoskeleton and cell membranes, result in ion fluxes and associated elastic and plastic deformations of tissue that themselves generate further stresses, ion flows and other biochemical changes (including, of course, gene activities), with reciprocal interactions. After an extended exploration of the information and programming metaphors, the subject may be returning to the themes of *Entwicklungsmechanik*; which would have pleased Driesch. □

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Trains of thought

John C. Marshall

Vehicles: Experiments in Synthetic Psychology.

By Valentino Braitenberg.
MIT Press: 1984. Pp.152.
\$14.95, £15.60.

WILLIAM Thomson, Lord Kelvin, is reputed to have remarked: "If you can make a mechanical model of it you understand it, and if you can't you don't". Kelvin was, of course, talking about the role of models in physics (and thinking about the success of his tidal wave predictor), but he could equally well have had psychological models in mind.

There is a tradition in theoretical psychology, extending back some two millennia, whereby the explanation of behaviour is explicitly equated with the construction of "toys" that exhibit the behaviour in question. Some of the outstanding contributors to this enterprise include Heron of Alexandria, who built a complex automaton wherein, on the lifting of a golden apple, a miniature Hercules shot a snake that then hissed; Albertus Magnus, whose *magnum opus*, a life-sized automatic servant, was destroyed, so legend goes, by Thomas Aquinas who suspected it to be the Devil's work; Jacques de Vaucanson, the constructor of an eating, drinking and quacking artificial duck, and of a flute-player that out-performed many human virtuosi; and the Jaquet-Droz (*père et fils*), whose dolls could write and draw with amazing facility. Like Magnus, the elder Droz and his models suffered a fate more extreme than failure to obtain tenure. Following a public exhibition of the writing-boy, Hermann von Helmholtz reminds us that "... this boy and its maker, being suspected of the black art, lay for a time in the Spanish Inquisition, and with difficulty obtained their freedom".

Closer to our own time, Grey Walter and Claude Shannon devised, respectively, tortoises and mice that ran mazes in electronic instantiation of the laws of classical learning-theory. To this distinguished line of artificial intelligentsia we must now add Valentino Braitenberg, a director of the Max Planck Institute of Biological Cybernetics at Tübingen.

Professor Braitenberg's *Vehicles* is precisely that; a series of 14 little vehicles, with surprisingly life-like properties. The sequence begins with a simple bug where one sensor is connected to one motor such that "the more there is of the quality to which the sensor is tuned, the faster the motor goes" and ends with creatures of more Rococo wiring that appear to demonstrate foresight, egotism and optimism. Each successive creature embodies the critical structures of all previous models and adds one further feature; the vehicles thereby illustrate a single line of evolu-

tionary ascent and painlessly introduce the unsuspecting reader to the basic concepts of self-regulating machines. Throughout, Professor Braitenberg writes in a dry, tongue-in-cheek fashion, poking sly fun at any philosopher (or Reith lecturer) whose hand is too unsteady to wield Occam's razor. He is thus a worthy successor to Julien Offray de la Mettrie, who in his *L'Homme Machine* of 1748, argued that

... since all the faculties of the soul depend to such a degree on the proper organization of the brain and of the whole body, that apparently they are but this organization itself, the soul is clearly an enlightened machine.

For both la Mettrie and Braitenberg, it's Chinese rooms all the way down.

In the main text, Professor Braitenberg's humour serves to illuminate his serious moral, "the law of uphill analysis and downhill invention", that is:

It is pleasurable and easy to create little machines that do certain tricks. It is also quite easy to observe the full repertoire of behavior of these machines — even if it goes beyond what we had originally planned, as it often does. But it is much more difficult to start from the outside and to try to guess internal structure just from the observation of behavior.

Worse,

it is actually impossible in theory to determine exactly what the hidden mechanism is without opening the box, since there are always many different mechanisms with identical behavior.

Accordingly, Professor Braitenberg provides an extensive appendix of biological notes on his vehicles. Here he sketches the analogies between his creatures and those of the given, natural world, leading one lightly into the complexities of current neuroanatomy and neurophysiology. The transition between the stark formalism of the hardware vehicles and the cell-synapse-circuit wetware that they mimic is mediated by a portfolio of phantasmagoric drawings by Maciek Albrecht.

For both amusement and enlightenment, *Vehicles* can be unhesitatingly recommended to anyone (student or teacher) involved in courses on "Brain and Behaviour". The volume would also make a marvellous Christmas present for any intelligent teenager interested in either computing or animal-watching, or better, both. □

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New in paperback

● *The Structure of Time* by W. H. Newton-Smith. Publisher is Routledge & Kegan Paul, price is £7.95, \$14.95.

● *Curious Naturalists* by Niko Tinbergen, a reprint of the revised (1974) edition of the book. Publisher is The University of Massachusetts Press, price is \$9.95.

● *Plant Biotechnology*, edited by S. H. Mantell and H. Smith, Vol. 18 in the seminar series of the Society for Experimental Biology. Publisher is Cambridge University Press, price is £12.50, \$19.95.

Sense of rays

Richard L. Garwin

Beam Weapons: The Next Arms Race.

By Jeff Hecht.

Plenum: 1984. Pp.363. \$17.95, £17.10.

IN FEW areas of science and politics has public interest grown as rapidly as in that of directed energy weapons, especially as applied to space defence and anti-satellite attack. Key to this surge of awareness is President Reagan's speech of 23 March 1983, in which he called for a research programme to "render nuclear weapons impotent and obsolete" by the ability to "intercept and destroy strategic ballistic missiles before they reached our own soil or that of our allies". Jeff Hecht's book was at the publisher's in draft form on that date, but the President's "Strategic Defense Initiative" (SDI) (or "Star Wars") speech figures prominently in the updated text.

With a degree in electronic engineering from California Institute of Technology, and seven years as managing editor of *Laser Focus*, Hecht is well placed to interpret for a popular audience the development and potential of beam weapons — that is, laser beams of infrared, visible or X-radiation; charged or neutral particle beams of protons, electrons or hydrogen atoms; and beams of microwave radiation. He makes a valiant attempt to report myth as myth and fact as fact. Unfortunately the jobs of popularizer and decision-maker alike are seriously impeded by an unusual lack of substantive unclassified material from the United States Government on beam weapons, and by the confusion between, on the one hand, the President's call for a total defence as a result of a long-term research programme and, on the other, the warmed-over silo defence which is being offered at least as a mid-term goal of the SDI.

Hecht does a good job of distinguishing between weapons and systems, noting the easy integration of target acquisition, aiming, fire and damage assessment in the eye, brain and arm of the human archer or marksman, as contrasted with a system in which these functions are dispersed over many satellites and centres of control and responsibility. He also covers well the technological side of the subject, describing the early high-power gas dynamic lasers, chemical lasers such as those which produce light from the reaction of hydrogen and fluorine, and excimer lasers now powered by electron beams. He emphasizes properly the degree to which plumbing and ducting is required for bringing the reacting gases to a chemical laser, but perhaps does not recognize adequately the degree to which similar considerations are important in venting gas from excimer lasers, because of the inevitable retention of some 90% of the pumping energy in the gas.

A further attractive feature of the book is Mr Hecht's tone of good-humoured scepticism, as in his observation that:

Pentagon officials have said that the Soviet effort [in laser weapons] is three to five times larger than the US program, an estimate that seems to hold constant even when the American budget is being sharply increased.

The treatment of the impact of space defence on national security is rather less solid. Hecht does not help to clarify matters by beginning with the "strategy of mutual assured destruction (MAD)" which, rather than being a strategy, is a fact of life. It is strategy to deter the opponent by threat of retaliation, but that one is able to do so and that one is deterred in return is a result of a technological fact — the destructiveness of nuclear weapons against societies — and not so much a matter of choice.

Strategic options flow from technological capability, and only to a certain extent can technology be created in the service of strategy. For instance, the technology suitable for defence against strategic ballistic missiles is for the most part even more suitable in the destruction of those same defences. Hence the ambivalence (almost to the point of schizophrenia) of those favouring space defence over the problem of where to base the defending weapons. As Hecht notes, Edward Teller explicitly recognizes that "it would be foolish to base a defensive system in space . . . because satellites are costly to put up and cheap to shoot down". A weapon that could destroy a booster in a few seconds might readily be destroyed by comparable weapons launched with the booster or already in space, and yet to keep weapons on the ground until the attack is in progress presents insuperable difficulties for intercept during boost phase. Mr Hecht dismisses such "pop-up defence" as unlikely even for mid-course intercept, but proponents of boost-phase defence and X-ray lasers argue valiantly that even a 180-s boost of an MX missile would be vulnerable to submarine-launched pop-up defence. Since the writing of this book, analysts have looked at countermeasures to some of the beam weapons, for instance fast-burn boosters to eliminate the threat of X-ray lasers or neutral-particle-beam weapons for boost-phase defence.

The issues surrounding beam weapons are at best murky and clear-thinking is not aided by statements of officials such as Dr George A. Keyworth, President Reagan's Science Adviser, who judges that:

. . . we can approach the debate about strategic defense as either optimists or pessimists and still come to the same conclusion: We must proceed. The optimistic view would have the United States be the first to develop effective defensive capabilities, giving us a persuasive negotiating posture for arms reductions . . . the pessimistic view sees the Soviets as the first to develop a real defensive capability . . . [Aerospace America, April 1984].

Whatever the persuasive power of this argument, it has no foundation in logic:

what about those technologists whose optimism is focused on penetration aids for defeating defences? No one doubts that starting from a conclusion one can find (some) arguments to support Keyworth's view, but in a democracy, and particularly when the questions involve not only hundreds of billions of dollars but the survival of nations, we need all the strength that open discussion and decision-making can provide. *Beam Weapons* will help to that end, together with the more extensive and technical *Space-Based Missile Defense* of March 1984 by the Union of Concerned Scientists, and especially the Background Paper by Ashton Carter, published in April 1984 by the Office of Technology Assessment.

In the book, Hecht displays a degree of constructive optimism and cites two possibly beneficial outcomes from beam weapons. One is "some sort of international peace-keeping organization to operate defensive satellites". The other is the hope that beam weapons "would tip the strategic balance of power to the defense", in providing missile defence, and "if the great powers can cope with the challenge of a transition to a strategic defense, the result could be a stabler world". I, however, would agree with the position taken by the French government in its presentation earlier this year (12 June) to the Committee on Disarmament in Geneva:

A situation in which each of the two principal powers sought to render its territory totally invulnerable, that is to say to escape any retaliation, without, however, being certain of being able to achieve this invulnerability, is fraught with danger:

On the one hand, the announcement itself of the intention to proceed with the creation of such systems constitutes itself an incitement to the acceleration of the offensive arms race: each power seeks to overcome (by saturation) the BMD [ballistic missile defence] systems envisaged by the other party, and in addition to increase greatly the number of non-ballistic carriers of nuclear weapons — notably cruise missiles.

Mr Hecht himself notes that the most credible role for a pop-up X-ray laser "is as part of a first-strike system, which would be put in place before the attack to defend against any enemy missiles which survived a massive first strike".

Beam Weapons is marred by misprints and by an irritating amount of repetition. Some references are quoted inaccurately, and the simple calculations provided would have been even more helpful had the arithmetical errors been corrected. But taken as a whole the book is a fair, readable attempt to make some sense out of the confusing claims and counterclaims about the feasibility and utility of directed energy in warfare. □

Richard L. Garwin is IBM Fellow at the Thomas J. Watson Research Center, Yorktown Heights, New York, and Adjunct Professor of Physics at Columbia University.

Spectroscopy of novel systems

From R.C. Thompson

Atomic spectroscopy is alive and well thanks to the continuing development of novel experimental techniques and their application to new areas of interest.

THOSE people who think that the subject of atomic spectroscopy was wrapped up several decades ago would have seen much evidence to the contrary at the recent EGAS conference held at Imperial College¹. Completely new areas of spectroscopy are still arising, bringing with them new and interesting physics. For example, the study of Rydberg atoms (atoms where one electron is excited into a state of high principal quantum number) has blossomed recently, and an up-to-date review of this field was given by C. Fabre (Ecole Normale Supérieure, Paris). Because many atomic properties scale as large powers of the effective principal quantum number n^* , Rydberg atoms can have large diameters (up to a fraction of a micrometre — large enough to be measured using a fine sieve), very long lifetimes and large polarizabilities. These facts make possible very unusual experiments — the detection of single microwave photons using Rydberg atoms as sensitive detectors; the modification of the spontaneous decay rate of atoms by putting them into a high-Q resonant microwave cavity; and even the possibility of constructing a single-atom laser (the usual many-body nature of laser action is made possible by the interaction of the atom with its images in the walls of the cavity). In experiments where a thermionic diode is used to detect ions formed by collisional ionization of laser excited Rydberg atoms, transitions to states with n up to 200 have been studied.

A relatively new area of spectroscopy was reviewed by G. Soff (Gesellschaft für Schwerionen, Darmstadt). Super-heavy quasi atoms are created for very short periods (10^{-20} s) in collisions between heavy projectile and target nuclei at energies of a few MeV per nucleon. As an example, at a total nuclear charge $Z = 164$ (formed in a Pb + Pb collision), the nucleus created has a radius of 10 fm, while the radius of the 1s electron wavefunction is 20 fm. Some unusual physical effects occur under such extreme conditions, such as the spontaneous emission of positrons during the collisions. This becomes energetically favourable at a combined charge $Z > 173$, when the 1s state enters the lower continuum of the Dirac Hamiltonian, that is, the binding energy becomes greater than the energy required for creation of an electron-positron pair. Measurements of

this and other effects in progress at GSI enable tests to be made of quantum electrodynamics in novel situations.

Another new development is the first observation of photon correlations in a true second-order two-photon decay (H.J. Duncan, University of Stirling). In this experiment, metastable hydrogen atoms (in the 2S state) in an atomic beam decay emitting two photons in random directions. Events are recorded where one photon enters each of two detectors placed 180° apart. Measurements of both the linear and circular polarization correlations between the two photons enable a test to be made of the celebrated Bell's inequality, which distinguishes the predictions of quantum mechanics from those of local realistic theories. As in other complementary experiments performed recently, these results favour quantum mechanics, but the controversy about whether or not quantum mechanics provides a complete picture still rages.

One of the highlights of the conference was the screening of a film showing the results of a series of calculations of the behaviour of an atom being irradiated by laser light (B.W. Shore, Imperial College London and Lawrence Livermore National Laboratory). The idealized two-level atom is a familiar concept, but here a slightly less idealized ten-level atom is treated, the results being displayed in the film as a two-dimensional plot of the density matrix of the atom as a function of

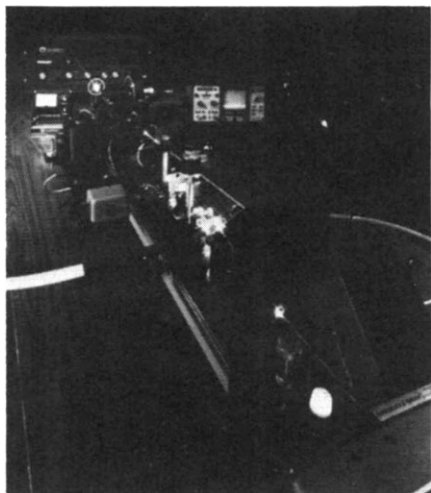
time. The phenomena illustrated include the periodic complete population inversion obtained with resonant coherent excitation and the gradual buildup of population in a specific excited level when the laser frequency corresponds to a multiphoton resonance. The film gave a valuable insight into the physical processes described by sets of equations treated usually only in an abstract mathematical manner.

Experiments using laser excitation were prominent at the conference and one striking example of the way the use of laser techniques is still growing is the application of coherent radiation (at wavelengths below 100 nm) generated by four-wave mixing in Kr gas to the study of VUV transitions in Xe, including the measurement of absolute wavelengths to 2 parts in 10^6 (T.J. McIlrath, University of Maryland and National Bureau of Standards, Washington).

Most laser experiments, however, are performed in more conventional wavelength ranges. The availability of reliable single-mode tunable dye lasers covering the whole of the visible spectrum has meant that high resolution and power are now easily achieved in this region. Numerous Doppler-free laser techniques have been developed for the recording of spectral lines under many different source conditions; the laser has thus become an invaluable aid to the study of the structure of atoms and molecules.

A recent trend is for much atomic spectroscopy to be performed at large centralized facilities such as accelerators (used for example for the production of beams of atoms in various states of ionization) and nuclear reactors (for the creation of rare isotopes). This places more stringent requirements on the reliability of experimental apparatus, so that there has been a move in general towards computer controlled equipment and automated data acquisition. The use of computers also in the analysis of data has meant that more complex atomic systems can be investigated and this complements the theoretician's growing ability to perform calculations on such systems.

The EGAS conference demonstrated the broad range of the subject, with sessions covering topics as diverse as term analysis, isotope shifts, electron spectroscopy and collisional broadening.



The Coherent 699-29 Autoscan, a self contained dye laser spectrometer ideally suited to high-resolution absorption spectroscopy. The heart of the system is the Coherent 699-21 actively stabilized single-mode ring dye laser. Control is provided by an Apple II microcomputer, which can also be used for up to three channels of data acquisition. Circle no. 100 for further details.

1. The Sixteenth European Group for Atomic Spectroscopy Conference was held at Imperial College, London on 10-13 July 1984.

R.C. Thompson is at the Division of Mechanical and Optical Metrology, National Physical Laboratory, Queens Road, Teddington, Middlesex TW11 0LW, UK.

Spectroscopy and spectrometry

Automation and computer-links are the main themes among the latest in spectrophotometers and spectrometers

● The CPS-240 six-cell positioner from Shimadzu enables the Shimadzu UV-240 spectrophotometer to display absorbance in a bar graph format, plus data as difference in absorbance multiplied by a factor for all or part number of the six cells. Also available is the capability to look at different wavelengths at each of the six cells and change the absorbance range for each. Temperature can be controlled over the range 16°–60°C, and a manual determination of which cell is being looked at is possible at any time.

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● A new economical system from Pye Unicam allows absorbance calibration for spectrophotometers in the ultraviolet and visible ranges. The set of four neutral-density filters provides a comprehensive check of absorbance linearity up to 2Å. Pye Unicam's original set of nine filters, calibrated up to 3Å, is still available for users needing a fully comprehensive check of absorbance linearity across the whole intensity range.

Circle No. 102 on Reader Service Card.

● The computerized D.B. scanning spectrophotometer UVIDEC-610/650 from Jasco International now incorporates software modifications to simplify routine analysis and enhance data presentation. A battery-backed memory stores data for at least two years, enabling users to save pre-selected parameter combinations for each of the six main methods already programmed into the computer. In addition, up to five sets of parameter/command instructions can be stored, so that routine or repetitive analysis may be performed at the touch of a key. The cathode-ray tube display includes a 'screen edit' function whereby users' comments are entered through the keyboard, stored in memory, and printed out as an integral part of the final report along with spectra and all other relevant data. Newly available computer accessories include double disk drive for data/spectra storage, and a full QUERTY keyboard for preparation of specific application programmes in BASIC or FORTRAN.

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● Newly introduced onto the US market, the low-cost infrared data station from Heyden Data Systems of North London — Spectrafile IR — is based on the Apple microcomputer. Spectrafile IR consists of three main modules: data acquisition and instrument control, data analysis and spectral search. The system provides operators with full control over an IR spectrophotometer with individual interfacing included in the system price.

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The Bio-Rad photometer for reading micro-titration plates.

● The Bio-Rad Model 2550 EIA reader is a photometer that automatically reads, with blanking and hard copy printout, a 96-well microtitration plate in less than 60 seconds. A digital absorbance-range display and plate-position indicator, as well as an operator error indicator and an absorbance overflow indicator, guide the user. Four high quality, narrow bandwidth interference filters, 405, 414, 450 and 492 nm, are supplied as standard equipment. Optional filters permit wavelength measurement at 380 to 700 nm. The reader has two measuring modes. The absorbance mode provides direct hard-copy printout of the absorbance values to three decimal places and matrix mode makes it possible to preselect an absorbance range, and automatically divide it into ten equal segments. The microprocessor assigns a value of 0–9 for each segment, and assay absorbance readings are catalogued into the appropriate range segment. All values are printed out in an 8×12 matrix that corresponds exactly to the layout of the 96-well microtitration plate.

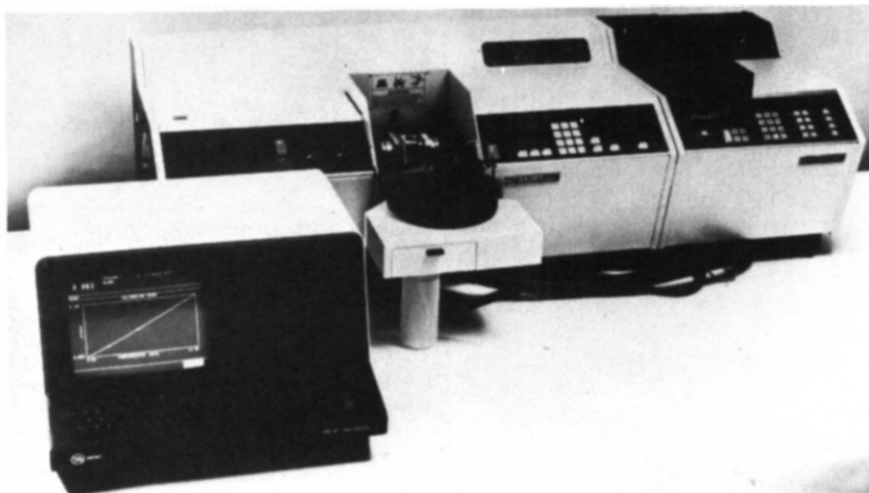
Circle No. 105 on Reader Service Card.

● The new range of Fourier transform spectrometers from Bomem, Inc. extends the operating wavelength range from the ultraviolet to the far infrared. A standard spectrometer with suitable detectors can produce spectra from 50,000 cm⁻¹ to 4 cm⁻¹. In addition to a UV to far-IR capability, the new spectrometers feature a fully evacuable spectrometer to eliminate water vapour; a five port sample compartment selectable from the control computer, and an automatic optical alignment system that ensures the system is always at peak efficiency. Bomem systems are available with a choice of resolutions, up to a maximum of 0.002 cm⁻¹.

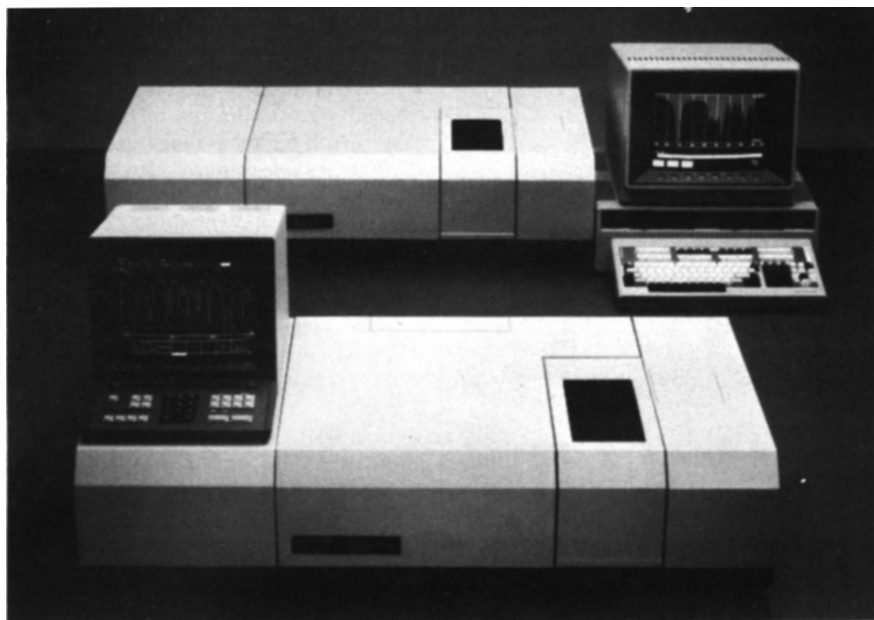
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● By using a new software package, the Varian DS-15 computer can be linked to an AA-1275 or AA-1475 spectrophotometer for complete atomic absorption data management. The DS-15 is a powerful desk-top computer with 12-inch CRT, 128 Kbytes RAM and two disk drives providing 720 Kbytes of storage. The single-beam AA-1275 and double-beam AA-1475 atomic absorption spectrophotometers are brought together by the new Varian software in a package specifically tailored to meet the needs of the busy analytical laboratory. No programming experience or typing ability is required. The operator is guided through a series of simple selections using menus and soft keys. Results are collected automatically when flame or furnace autosamplers are used but samples may also be presented manually. Flexible report generation allows reports to be tailored to individual needs. The operator may choose the amount of data to be printed and the format of the report. Data from flame, furnace and hydride analyses may be combined in a single report.

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Varian's AA-1475 spectrophotometer linked to the DS-15 data station.



The 1700 Series Fourier transform infrared spectrometer from Perkin-Elmer.

● Based on a single high-performance interferometer, Perkin-Elmer's 1700 Series of Fourier transform infrared spectrometers is a new range of compact, mid-infrared instrumentation. The instruments in the 1700 Series differ in their data handling capabilities. The spectrometer itself uses an improved Michelson interferometer designed to compensate internally for any effects of dynamic misalignment, providing an extremely stable system resistant to vibration and disturbances during a scan. Interferometer alignment is very simply carried out by a closed-loop servo system and is initiated from the control console. The standard spectrometer has a frequency range of $4,400\text{ cm}^{-1}$ to 400 cm^{-1} and a choice of resolution down to 2.0 cm^{-1} . Resolution can be increased to 1.0 cm^{-1} by the addition of an optional memory board.

Circle No. 108 on Reader Service Card.

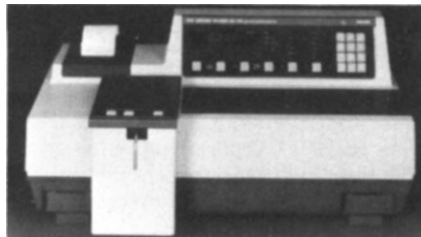
● The new GN Series NMR spectrometers from General Electric incorporate a 293D pulse programmer along with the GN series data system and NMR software which enables the user to program a wide variety of pulse sequences. This feature, together with the ability of the instrument to vary phase shifts from 0 to 360° , makes it possible to perform most types of multi-pulse, multiple-quantum NMR experiment. In addition, the programmable pulse modulation and phase shift capabilities of the GN Series also make it possible to perform all the 2-D NMR experiments currently in use. The GN Series system incorporates an easy-to-use multiple-level, menu-driven software package which includes automatic shimming, locking, gain, spectral phasing and integration programs.

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These notes are based on information provided by the manufacturers. For further details circle the appropriate numbers on the Reader Service Card bound inside the journal.

● The new Nicolet 20DXB and 20SXB FT-IR spectrometers are based on field-proven Nicolet FT-IR technology. The 20B Series performs with a maximum resolution of 0.5 cm^{-1} . Resolution is continuously selectable from 0.5 cm^{-1} to 64 cm^{-1} . The spectrometer can scan at rates as fast as 4 per second, making it suitable for monitoring dynamic processes such as capillary GC-IR, catalysis, and polymer curing. An external collimated beam is standard for 20SXB GC-IR interface and remote detector experiments. A computer-controlled flip mirror allows fast, easy, precisely pre-aligned switching between the two sample path geometries. Designed for the chromatographer, the new 20SXB GC-IR software provides simplified, automated operation, from real-time data collection and display to identification of components.

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For industrial and teaching laboratories, the Pye-Unicam PU8600 spectrophotometer.

● Powerful microprocessor features and a high-performance optical system are combined in Pye Unicam's new generation of compact UV/vis spectrophotometers. The PU8600 Series of microprocessor/keypad operated instruments comprises two main versions — the PU8600, suited to the majority of industrial and teaching applications, and the PU8610, optimized for the special requirements of life scientists. The PU8600 Series is modelled on the popular SP6 Series, which remains in production for users requiring a simpler machine.

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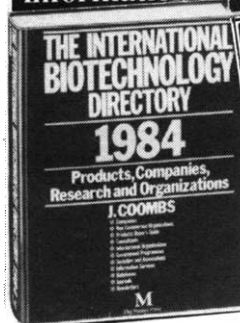
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• Spectrafile-UV is an ultra-violet spectrophotometer data station which collects, plots and manipulates UV data. Features include full instrumental set-up and control. Spectrafile-UV allows data to be collected and displayed and stored on disk. Data can be manipulated with the functions: add, subtract, multiply, integrate, differentiate, normalize, smooth and others. Customized routines for kinetic studies are also available and Spectrafile-UV, from Heyden Datasystems, interfaces to any UV/VIS spectrometer.

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• Sirius 100 and Cygnus 25 are FTIR spectrometers from Mattson Instruments Inc. They offer resolution capabilities of 0.125 wavenumbers and 0.5 wavenumbers respectively over the mid-infrared spectral range with optional range extensions to 25,000 wavenumbers and 10 wavenumbers. Notable features of both spectrometers are high-throughput 2-inch diameter optics, a Michelson interferometer based on cube-corner retroreflectors for high dynamic stability (no air bearings), and a large accessible sampler chamber.

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• The STARLAB data system available from Glen Creston of Stanmore, Middlesex, is a UNIX-based software system with multi-user/multi-tasking capability and 32-bit arithmetic. Data storage capacities from 10Mb to 340Mb are provided by Winchester disks, and back-up storage for data and software by 8-inch floppy disks is standard. Spectrometer operation and data manipulations are commanded through a standard graphics terminal.

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